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Haem Oxygenase -1 induction and activity in astrocytes and possible interactions with nitric oxide

By

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A thesis submitted as partial fulfilment for the degree of Doctor of Philosophy in the Faculty of Science at the University of London

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Abstract

Haem oxygenase-1 (HO-1) is the inducible form of the enzyme responsible for the breakdown of haem, metabolising it to carbon monoxide (CO), bilirubin and iron. Protective properties have been attributed to HO-1 induction and haem breakdown products. Upregulation of HO -1 has been reported in many neurodegenerative disorders and it has been postulated that this may play an important role in the stress defences of the cell. Inducible nitric oxide (iNOS) activity, nitric oxide production and mitochondrial dysfunction are also reported in neurodegenerative disorders and it has been shown that inducers of iNOS also induce HO-1. Rat astrocytes have been used to examine possible interactions between the iNOS/HO systems by inducing iNOS activity with IFNγ/LPS and HO-1 with hemin. It was confirmed that IFNγ/LPS mediated NO production caused an upregulated HO-1 expression and an increase in HO activity. Further, it was shown that hemin treatment was able to prevent the previously reported NO mediated irreversible inhibiton of complex IV of the mitochondrial electron transport chain seen when astrocytes are treated with IFNγ/LPS. Hemin plus IFNγ/LPS treatment was accompanied by an increase in HO activity and an increase in glutathione (GSH) levels and it is postulated that together these confer protection.

It may be concluded from this thesis that there are complex interactions between HO and NOS which could contribute to the ability of astrocytes to provide protection to neurones against oxidative and nitrosative stress. This may be relevant to mechanisms by which neurodegeneration occur.

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Contents

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List of abbreviations

AD	Alzheimers disease
ALS	Amylotrophic lateral sclerosis
ATP	adenosine triphosphate
BH4	tetrahydrobiopterin
BSA	bovine serum albumin
L-BSO	L-buthionine – (S,R) – sulfoximine
cGMP	cyclic 3':5 – guanosine monophosphate
CNS	central nervous system
CO	carbon monoxide
CoQ1	ubiquinone
CP motif	cysteine-proline motif
CS	citrate synthase
DAPI	4'6-Diamidino-2-phenylindole
DETA-NO	(z)-1-[2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DMSO	dimethyl sulphoxide
DTNB	5,5' dithio-bis-(nitrobenzoic acid)
EBSS	Earle's balanced salt solution
eNOS	endothelial nitric oxide synthase
ETC	electron transport chain
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
FITC	flourescein isothiocyanate

GCL	glutamate-cysteine ligase
GFAP	glial fibrillary acidic protein
GSH	reduced glutathione
HO	haem oxygenase
H2O2	hydrogen peroxide
HBSS	Hank's balanced salt solution
HPLC	high performance liquid chromatography
HSP32	heat shock protein 32
IL – 6	interleukin - 6
IL – 10	interleukin - 10
IL - 1β	interleukin - 1β
IFNγ	interferon γ
iNOS	inducible nitric oxide synthase
LDH	lactate dehydrogenase
LDL	low-density lipoproteins
L-NIL	L-N6-(L-iminoethyl) lysine hydrochloride
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MEM	minimal essential medium
MS	multiple sclerosis
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	nicotinamide dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NFT	neurofibrillary
nNOS	neuronal nitric oxide synthase

NO	nitric oxide
O2•-	superoxide
$OH^{\cdot}$	hydroxyl radical
ONOO-	peroxynitrite
PBS	phosphate buffered saline
PD	Parkinson's disease
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	sodium nitroprusside
SnPPIX	tin protoporphyrin IX
SOD	superoxide dismutase
TNFα	tumor necrosis factor α
ZnPPIX	zinc protoporphyrin IX

Chapter 1.

Introduction

In recent years, study of Haem oxygenase (HO) (EC 1.14.99.3) the enzyme that metabolises haem to carbon monoxide (CO), iron and biliverdin, has led to the suggestion that the inducible form is important in the antioxidant defences of the cell. The mechanisms of protection are not fully understood, indeed it appears in some cases that induction of HO can actually prove harmful. Expression of inducible HO is documented in many disease states including those that cause neurodegeneration (Schipper, 2004).

This thesis has investigated the effects of induction of HO in rat astrocytes and its interaction with inducible nitric oxide synthase (iNOS)/nitric oxide (NO). This chapter will introduce haem and the HO family of enzymes, examine the pro and antioxidant effects that induction of HO can cause, then go on to discuss oxidative stress and the roles of HO and NO in neurodegeneration. Interactions between the iNOS/HO-1 enzyme systems will be examined and their relevance to neurodegeneration discussed. Finally the aims of the thesis will be explained.

1.1 Haem

Haem (**Figure 1.1**.), also known as iron protoporphyrin IX, is a member of the porphyrin family of compounds. Porphyrins are cyclic tetrapyrroles in which the four pyrrole rings are attatched through four methylene bridges. Porphyrin molecules alone do not bind O_2 nor participate in enzyme catalysed reactions, these properties are conferred on the porphyrin molecule by the chelation of multi-valent transition metals such as iron, tin or zinc and attachment of the

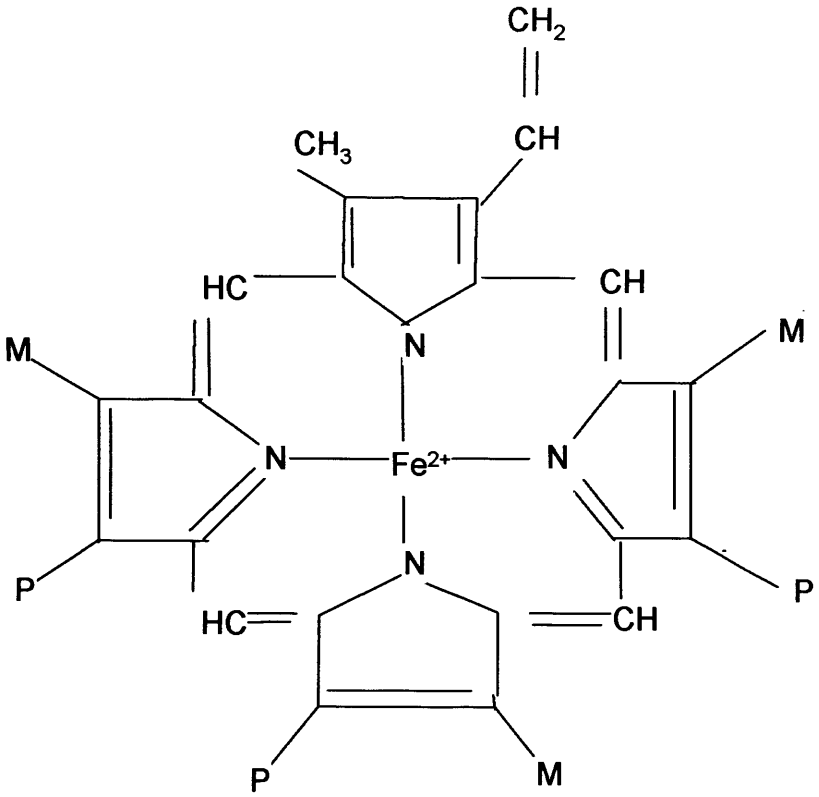


Figure 1.1. The structure of haem

M = methyl group, P = propionyl group.

(adapted from Scheffler, 1999)

protein moiety (Chang *et al.*, 2005). When iron is chelated by the porphyrin haem is the resulting molecule. When ferric protoporphyrin IX binds a hydroxyl ion it is called hematin; if it binds a halide or acid anion it is hemin (Maines, 1984). The site of haem synthesis is split between the cytoplasm and the mitochondria. Synthesis starts and finishes in the mitochondria with all intermediate steps taking place in the cytoplasm (**Figure 1.2**.). The capacity of the chelated Fe ion to undergo a reversible change in the oxidation state ($Fe^{2+} \leftrightarrow Fe^{3+}$) makes haem compounds effective biological catalysts. Haem promotes most biological oxidation processes and is therefore important in cellular homeostasis. Haem serves as a prosthetic group for proteins involved in oxygen transport, mitochondrial respiration, drug metabolism, steroid biosynthesis, cellular antioxidant defenses, and signal transduction (Maines, 1997)

Free haem does not accumulate normally in tissue, this occurs only under pathological conditions (Maines, 1988). The term 'free haem' refers to the molecule before it is incorporated into haemoproteins, or when these proteins are being broken down.. Free haem is quite hydrophobic, readily enters cell membranes, and can greatly increase cellular susceptibility to oxidant-mediated killing (Jeney *et al.*, 2002). Indeed it has been shown that free haemoglobin in plasma when oxidised can provide haem to endothelium which can enhance cellular oxidant mediated injury (Jeney *et al.*, 2002). Free haem is always available, due to the constant breakdown of haemoglobin containing haem from erythrocytes and other enzymes using haem as a prosthetic group. Transporting heme via the circulatory system would expose the system to excessive toxicity via free radical production and oxidative stress and this may be the reason why

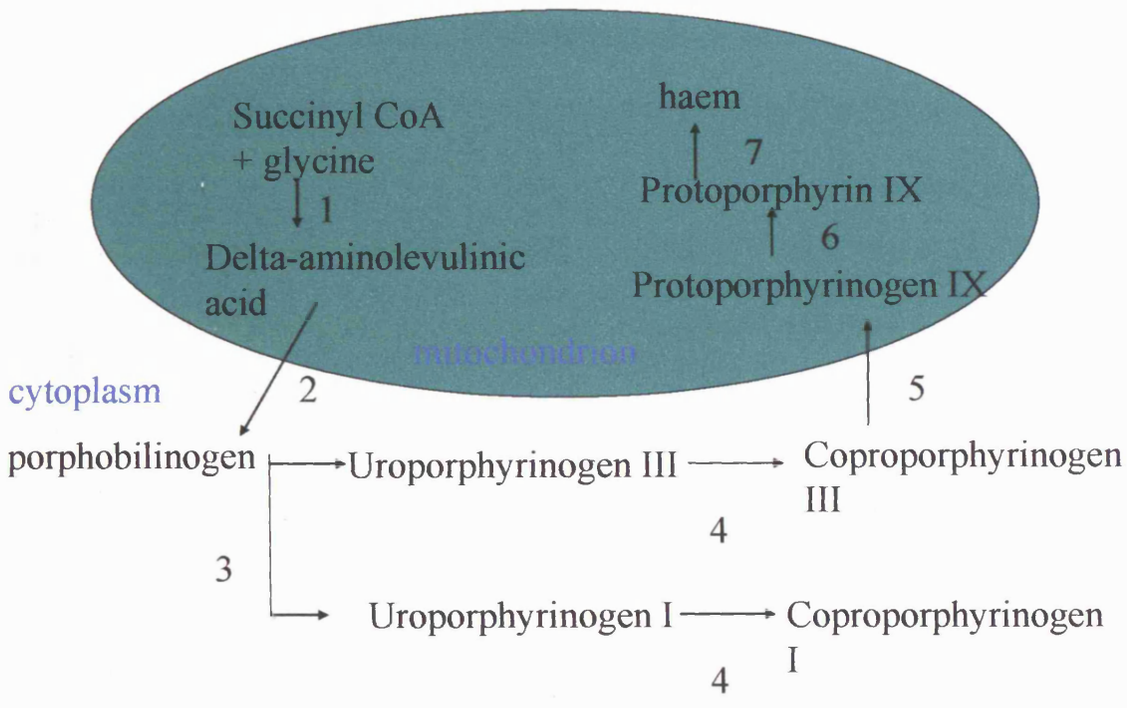


Figure 1.2. Haem synthesis pathway

Enzymes are, **1**. delta-aminolevulinic acid synthetase, **2**. delta-aminolevulnic acid dehydratase, **3**. uroporphyrinogen I synthase and urophyrinogen III cosynthase, **4**. uroporphyrinogen decarboxylase, **5.** coproporphyrin III oxidase, **6**. protoporphyrinogen IX oxidase, 7. ferrochetalase.

there is a requirement for haem degradation rather than a mechanism of haem reutilization (Sassa, 2004).

1.2. **The haem oxygenase family of enzymes**

In 1964 the enzymatic degradation of haem to bilirubin was first demonstrated *in vitro* by Wise & Drabkin, (1965). This finding was confirmed by Tenhunen *et al.*, (1968) who went on to characterise the enzyme and its cellular location. Interest in haem oxygenase as the enzyme was named, was rekindled in the 1980s when an inducible form, named HO-1 was discovered (Keyse & Tyrell, 1989) which was identical to heat shock protein 32 (hsp 32) and caused interest in HO as a stress protein. It was noted that HO-1 appeared to be induced by various forms of oxidative stress and it was proposed that induction of the enzyme provided cellular protection against these insults (Stocker, 1990). To add further weight to this theory, both haem and HO are highly conserved molecules across almost all forms of life (Morse & Choi, 2002) and molecules so evolutionarily conserved and ubiquitous usually serve a fundamental purpose. The HO reaction involves the oxidation of haem to biliverdin, carbon monoxide (CO) and free iron. Biliverdin is subsequently reduced to bilirubin by the action of biliverdin reductase (EC 1.3.1.24).The reaction requires O_2 and reduction of haem iron from Fe^{3+} to Fe^{2+}. Nicotinamide adenine dinucleotide phosphate (NADPH) is the source of the reducing equivalent (Maines, 1997). (Figure 1.3). To date there have been 3 isoforms of HO described. The isozymes are different gene products and also differ in

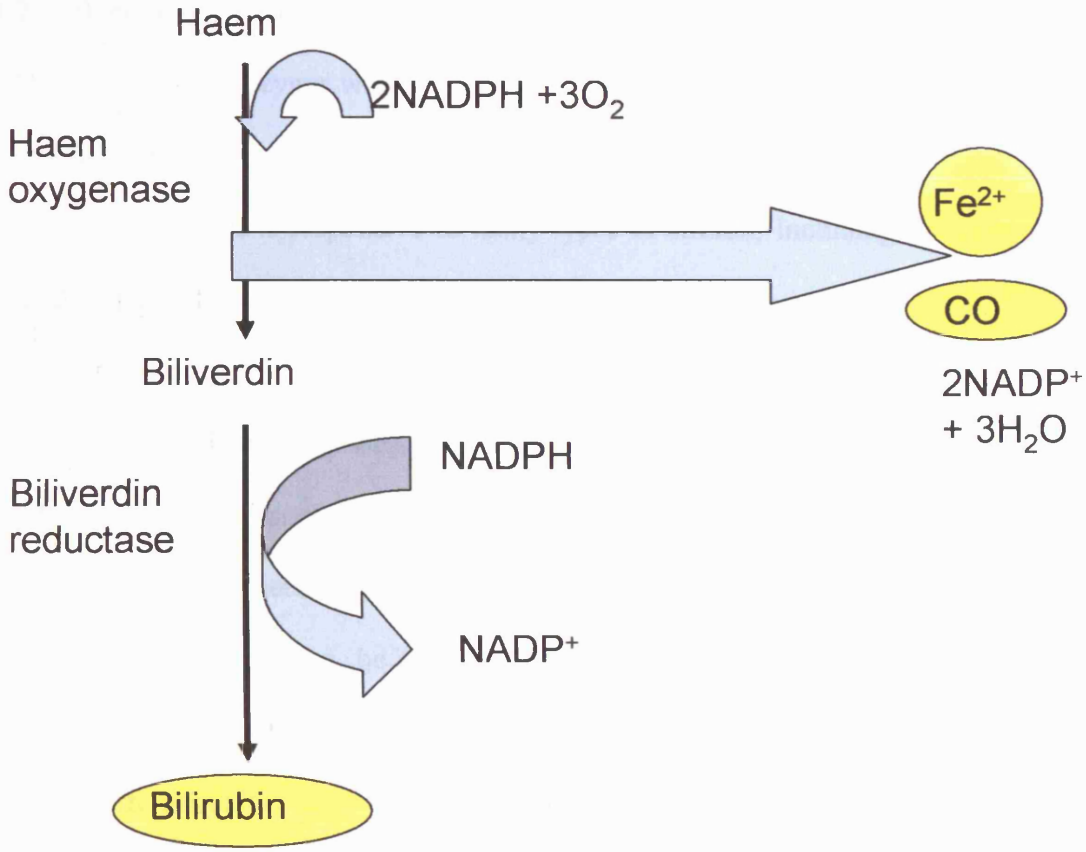


Figure 1.3. The haem oxygenase reaction

nucleotide sequences, amino acid composition, transcript number and size (Cruse & Maines,1988).

1.2.1. Haem oxygenase – 1

HO-1 is the HO isozyme which will be studied in this thesis. As mentioned previously HO-1 was identified as hsp-32 in 1989, and since that time it has become clear that it is responsive to many types of stimuli, including all those stimuli capable of causing oxidative stress (Maines, 1997). In normal unstressed conditions the spleen is the only organ in the body where HO-1 is the predominant isoform expressed, due to the constant breakdown of senesent erythrocytes (Braggins *et al.*, 1986). The protein has 288 amino acids and a molecular mass of between 30,000 and 33,000Da (Shibahara *et al.*, 1985). Expression is thought to be mainly regulated at the transcriptonal level (Rizzardini *et al.*, 1993; Choi & Alam, 1996; Durante *et al.*, 1997; Fogg *et al.*, 1999). Differences do exist though between species in HO-1 regulation, for example the rat HO-1 promoter has a heat shock responsive element which is not functional in the human gene (Okinaga *et al.*, 1996). However for rat, mouse and human, the extent of homology for HO-1 is greater than 80%. Neither human or rat HO-1 contain any cysteine residues (Shibahara, 2003). Poss & Tonegawa (1997) report that HO-$1^{-/-}$ mice were unable to produce viable litters and showed partial prenatal lethality when crossed with heterozygotes. Surviving mice develop progressive inflammatory disease characterised by splenomegaly, lymphadenopathy, leukocytosis, and hepatic and renal inflammation. In addition the animals display increased iron accumulation and are more sensitive to oxidative stress. One case of human HO-1 deficiency has been reported (Yachie

et al., 1999) with similar clinical features to the HO-1 knockouts, however in the case of human HO-1 deficiency it appears that there is even more sensitivity to oxidative stress and the patient in this case died at age 6 (Kawashima *et al.*, 2002). It is worth noting that the mother of the child in this case had experienced two intrauterine deaths making it likely that mutations of the haem oxygenase gene are generally lethal in utero (Kawashima *et al.*, 2002).

1.2.2. Haem oxygenase – 2 & 3

HO-2 is a constitutive isozyme and is the major HO isoform found in all organs (except as mentioned in spleen). Unlike HO-1 it is only responsive to adrenal glucocorticoids (Maines, 1997). Human HO-2 has 316 amino acid residues and a molecular mass of 36000Da and contains 3 cysteine residues (Shibahara, 2003). Unlike HO-1, HO-2 contains 2 copies of the haem binding site, a dipeptide of cysteine and proline (CP motif) which are not involved in haem breakdown. These CP motifs are conserved at equivalent positions in human, rat and mouse HO-2 (Shibahara, 2003). This indicates that HO-2 may function as a 'haem regulator', binding haem to maintain the intracellular haem level or to prevent haem from causing oxidative stress. Erythroid type δ-aminolevulnic acid synthase also contains the CP motifs (Lathrop & Timko, 1993), suggesting a possibility that haem itself may regulate its degradation and synthesis. HO-2 knockout mice show a milder phenotype than HO-1 knockouts with no noticeable morphological alterations. H0-2 deficient mice are fertile (Poss *et al.*,1995) but HO activity is markedly reduced in the brain and males show ejaculatory abnormalities and reduced mating behaviour (Burnett *et al*, 1998) and

they show increased susceptibility to hyperoxic lung damage (Dennery *et al.*, 1998).

McCoubrey *et al*, (1997) reported the existence of a third isoform, HO-3 in the rat in 1997, in organs including the spleen, liver, kidney and brain. They reported a tissue distribution similar to that of HO-2 . The predicted amino acid structure of HO-3 was different to HO-1 and HO-2 but with a 90% sequence homology to HO-2 and only marginal HO activity (0.4 nmol bilirubin/min/mg protein compared to specific activities of 107 and 67 nmol bilirubin/min/mg protein for purified rat hepatic HO-1 and testicular HO-2 (Trakshel *et al.*, 1986), respectively). A recent report (Hayashi *et al.*, 2004) has suggested that HO-3 is not in fact expressed in rat tissues and that there are no functional HO-3 genes in rat. Hayashi *et al.*, (2004) set out to examine the structure of the rat HO-3 gene and detect any possible HO-3 transcripts and products in rat, and although they did discover two HO-3 genes, they concluded that they are in fact pseudogenes, which are not functional.

1.3. The products of haem breakdown

The haem oxygenase reaction as mentioned previously metabolises haem to CO, iron and biliverdin which is rapidly reduced to bilirubin by biliverdin reductase.

1.3.1 Carbon Monoxide

Historically this molecule has been thought of as a toxin and first came to the attention of biologists in 1857 when Claude Bernard described the affinity of CO for haemoglobin (Bernard, 1857, cited in Otterbein & Choi, 2000). This

prompted research that placed CO in the category of poisons and toxins. Toxic effects of CO are caused by its ability to interact with iron, which is co-ordinated at the centre of all haem proteins (Piantadosi, 2002). CO can then inhibit the proteins ability to bind O_2 (e.g. haemoglobin) or inhibit oxidation-reduction reactions requiring the transfer of electrons through the haem moiety (e.g. cytochromes) (Piantadosi, 2002). CO can also bind to proteins that contain other transition metals at the active site e.g., copper and can interfere with their functions (Piantadosi, 2002).

In 1963 the physiologist Ronald Coburn demonstrated that CO is produced by the bodies of humans and other animals (Coburn *et al.*, 1963) and during recent years , research has shown that although CO at high concentrations is certainly lethal, low concentrations of this molecule can exert very different effects on physiological and cellular functions. For example, endogenous production of CO has been measured in cultured olfactory receptor neurons at ~ 4.7 pmol/mg protein (Ingi & Ronnett, 1995) and concentrations of between 50 and 500ppm CO have been shown to be cytoprotective against hyperoxia in rodents (Otterbein et al., 1999). Investigations have shown that low concentrations of CO can exert numerous biological functions such as neurotransmission (Maines, 1993; Snyder *et al.*, 1998), protection against cell death (Brouard *et al.*, 2000) anti-inflammation (Otterbein *et al.*, 2000), protection against oxidative injury (Otterbein *et al.*, 1999), inhibition of cell proliferation (Morita *et al.*, 1997; Song *et al.*, 2002) and tolerance of organ transplantation (Sato *et al.*, 2001; Song *et al.*, 2003). Biological functions akin to that of nitric oxide (NO) have been ascribed to endogenously produced CO in that it is able to bind to soluble guanylate

cyclase thereby effecting the activation of cyclic 3': 5'-guanosine monophosphate (-cGMP). It could be argued, that because the capacity of NO to activate guanylate cyclase is 30-fold more effective than CO (Stone & Marletta., 1994), it is not important in this area. However CO is a very stable molecule and can accumulate in the cell because it reacts exclusively with haem (Otterbein & Choi, 2000). The capacity of cells in the brain to generate CO is much greater than that to generate NO (Maines, 1997). Furthermore the simultaneous presence of NO and CO favourably affects binding of CO to haem (Maines, 1997). CO also exerts its effects via other pathways such as the mitogen activated protein kinase (MAPK) pathway, which involves phosphorylation cascades responsible for transducing inflammatory signals from the cell surface through to the nucleus, which could result in cellular activation and production of pro-inflammatory cytokines which amplify inflammation. CO has been shown to downregulate production of some pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, and at the same time upregulate production of the anti-inflammatory cytokine IL-10 via the p38 and JNK MAPK pathways (Otterbein *et al.*, 2000; Morse *et al.*, 2003). It has also been shown that the anti-apoptotic action of CO is mediated via the p38 MAPK system (Brouard *et al.*, 2000).

1.3.2 Bilirubin and biliverdin

Bilirubin is known as a potentially toxic agent, which may accumulate in the serum of neonates causing jaundice (Otterbein & Choi, 2000). In high concentrations it can deposit in selected brain regions and cause neurotoxicity (Gourley, 1997). However bilirubin is also the most abundant endogenous antioxidant in mammalian tissues and accounts for the majority of the

antioxidant activity of human serum (Gopinathan *et al.*, 1994). It has been reported that under low oxygen concentrations (2%) and when incorporated into liposomes, it scavenges peroxy radicals as efficiently as α-tocopherol (Stocker *et al.*, 1987). Numerous studies have attributed protective effects to bilirubin. (Gopinathan et al., 1994; Kato et al., 2003; Sedlak & Snyder 2004). Higher baseline serum bilirubin levels have been reported to correlate with a lower incidence of myocardial infarction in men, maybe due to the inhibitory effect of bilirubin on the oxidation of low-density lipoproteins (LDLs) (Djousse *et al.*, 2003). In the laboratory, Dore *et al.*, (1999) report protection of neurons against oxidative stress and Clark *et al.*, (2000) report cytoprotection of smooth muscle cells by HO-1 derived bilirubin. Yamaguchi *et al.*, (1996) report that bilirubin acts as a scavenger via its own oxidation to scavenge reactive oxygen species (ROS) produced by ischemia-reperfusion in the liver. Each molecule of bilirubin that acts as an antioxidant is itself oxidised to biliverdin which is immediately reduced back to bilirubin by biliverdin reductase (**Figure 1.4**.). Cell lines which are designed to express less biliverdin reductase have been reported to exhibit increased vulnerability to oxidative stress (Baranano *et al.*, 2002). In addition, Biliverdin has been found to have anti-viral activity (Morse & Choi, 2005) and has also been shown to provide protection in a rat model of ischaemic injury (Vachharajani *et al.*, 2000).

1.3.3 Iron

Free iron is released into the endoplasmic reticulum by haem breakdown. Iron is known to take part in Fenton chemistry, and generate the highly damaging hydroxyl radical (•HO) by acting as a catalyst for the Haber-Weiss reaction:

$H_2O_2 + O_2^- \longrightarrow O_2 + {}^-OH + {}^\bullet OH$ **(1.1)**

This reaction as written is exceedingly slow, with a reaction rate of 3.0 M^{-1} s^{-1} at neutral pH (Haber & Weiss, 1934). However iron causes the reaction to occur much more quickly:

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^-OH + {}^\bullet OH$ **(1.2)**

Fe^{3+} + ascorbate $\longrightarrow$ Fe^{2+} + ascorbate radical **(1.3)**

$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2$ **(1.4)**

The rate constant for equation **(1.2)**, which is known as the Fenton reaction, for EDTA chelated iron is $10^4 M^{-1}$ s^{-1} at pH 7.0 (Sutton & Winterbourne, 1984). The rate constant for equation **(1.4)** when the iron is chelated with EDTA has been determined to be $10^6 M^{-1}$ s^{-1} at pH 7.0. (Butler & Halliwell, 1982).

Thus free iron could and is known to have pro-oxidant effects on the cell. These effects include stimulation of membrane lipid peroxidation and causing sensitivity to other oxidants (Aust *et al.*, 1985, Balla *et al.*, 1990). However release of iron by the HO catalysed reaction results in the upregulation of ferritin, the iron storage protein (Eisenstein *et al.*, 1991). This upregulation has proven to be cytoprotective in a model of oxidative stress (Balla *et al.*, 1992) and Otterbein *et al.*, (1997) have shown that when iron is chelated by the exogenous iron desferoxamine, no ferritin is induced and protection is ablated.

1.4. Pro and antioxidant effects of HO induction

Increased levels of HO-1 expression are seen in many disease states, not only those involving haem catabolism. For this reason many studies have been made on the induction and inhibition of HO expression and activity in many cells and

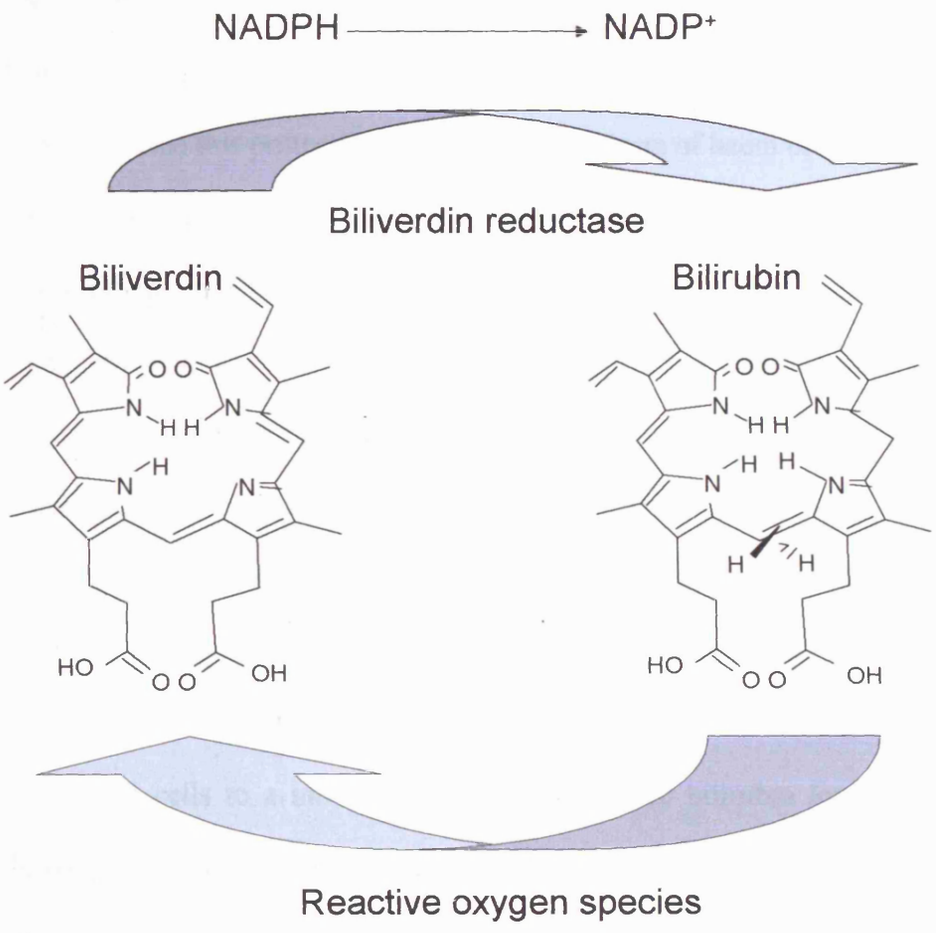


Figure 1.4. The biliverdin-bilirubin cycle

Lipophilic reactive oxygen species act directly on bilirubin, leading to its oxidation to biliverdin. Biliverdin reductas catalyses the reconversion of biliverdin to bilirubin permitting bilirubin to detoxify a 10,000-fold excess of oxidants. (Adapted from Sedlack & Snyder, 2004).

tissues both *in vivo* and *in vitro*. It has become apparent that the effects of HO activity and expression can be varied and not always entirely protective.

1.4.1. Protective effects of HO induction

Many of the studies that have found a protective role for HO expression/activity have attributed this protection to one of the products of haem breakdown, mainly bilirubin and CO. For example HO derived bilirubin has been found to be protective in a number of ways. In a rat model of cardiac ischaemia reperfusion injury bilirubin was shown to restore myocardial function and minimise both infarct size and mitochondrial damage (Clark *et al.*, 2000). Micromolar concentrations of bilirubin used to rinse murine liver and cardiac grafts during ex-vivo organ reperfusion or preceeding *in vivo* transplantation attenuated biliary dysfunction and cell injury of rat livers (Kato *et al.*, 2003). This protection mimicked the effect of HO-1 preconditioning of the livers. Exposure of aortic endothelial cells to a medium containing increased bilirubin levels which was derived from hemin treated cells markedly potentiated HO-1 expression and enzyme activity induced by NO donors (Foresti *et al.*, 2003). This suggests that signalling actions of NO that are related to the induction of HO are affected by haem metabolites such as bilirubin and so increase protection (in this case) of vascular epithelium against nitrosative stress. CO has been shown to be responsible for arterial vasodilation through activation of guanyl cyclase (Morita *et al.*, 1995; Morita & Kourembanas, 1995; Sammut *et al.*, 1998). Haem which potently induces HO-1 has been shown to lower blood pressure in spontaneously hypertensive rats (Levere *et al.*, 1990) and this is suggested to be mediated by CO. Expression of HO-1 has also been implicated in cellular protection by virtue

of its expression alone . Taylor *et al.*, (1998) found that induction of HO-1 in the rat lung protected against hyperoxia, but that this protection was still seen even when HO activity was inhibited. There is a possibility that ferritin which is co-induced with HO-1 and acts to sequester iron is responsible for this protection, however it has been reported that HO-1 induction is not necessary for ferritin induction (Balla *et al.*,1995) so the mechanism for protection here is not entirely clear.

1.4.2. Possible Harmful effects of HO induction

The HO reaction can itself potentially generate hydrogen peroxide (H_2O_2), as a significant metabolic side product, as demonstrated by Noguchi *et al.*, (1983). Thus HO activity is a possible source of $^{\cdot}OH$ because both H_2O_2 and Fe^{2+} are released in the same microenvironment. *In vitro* studies have suggested that under certain conditions HO activity may potentiate, rather than attenuate oxidant toxicity. For example, Da Silva *et al.*, (1996) found that inhibiting HO activity could actually attenuate toxic effects of an H_2O_2 challenge after a short term oxidative stress of epithelial cells, but a longer term incubation with hemin effectively protected against a later H_2O_2 challenge. Another study found that concurrently treating astroglial cells with inhibitors of HO activity such as Sn mesoporphyrin protected the cells against the cytotoxic effect of H_2O_2 treatment (Dwyer *et al.*, 1998). Overexpression of HO-1 has also proved damaging in some cases. Suttner & Dennery, (1999) found that overexpressing HO in hamster fibroblasts proved cytoprotective only with low HO activity (less than five-fold compared to basal levels) however higher activity was correlated with significant

oxygen cytotoxicity. It was concluded that this was due to increased levels of reactive iron.

1.5. Oxidative stress in the brain

Oxidative stress occurs when oxygen free radicals are generated in excess through the reduction of oxygen. Reactive oxygen species (ROS) consist of oxygen free radicals and associated entities that include superoxide ($O_2^{\cdot-}$) hydrogen peroxide (H_2O_2), singlet oxygen, nitric oxide (NO), and peroxynitrite ($ONOO^-$). Some of these species are produced at low levels under normal physiological conditions and are effectively scavenged by a number of antioxidant molecules such as superoxide dismutase (SOD) glutathione, catalase and small molecules such as vitamins C and E (Chong *et al.*, 2005) The superoxide radical is the most frequently occurring radical that produces H_2O_2 by dismutation. From this reaction it is then possible for the hydroxyl radical ($OH^{\cdot}$)to be produced by the Haber-Weiss reaction (**Equation 1.1**) in the presence of ferrous iron (Chong *et al.*, 2005). It is also possible for the $OH^{\cdot}$ radical to be produced through an interaction of the superoxide radical and NO (Fubini & Hubbard, 2003). In this reaction NO reacts with superoxide which leads to the generation of peroxynitrous acid, and spontaneous decomposition of this acid forms the hydroxyl radical (Fubini & Hubbard, 2003). Indeed although NO is included as an oxygen free radical, it and other NO species have become the subject of numerous studies as reactive nitrogen species (RNS) due to their implication in the pathogenesis of many diseases.

Oxidative stress occurs in the brain when the ability of the endogenous antioxidants is overridden by excess production of ROS/RNS. The brain is highly susceptible to oxidative stress for a number of reasons. Despite constituting 2% of body weight, the brain is known to possess the highest oxygen metabolic rate of any organ in the body (Maiese, 2002 in Ramachandran, 2002), and consumes about 20% of the total amount of oxygen in the body. This high metabolic rate leads to an increased likelihood of free radical generation over and above that with which endogenous defenses are able to deal. In addition to this brain tissues contain high amounts of unsaturated fatty acids which can be attacked by ROS (Chong *et al.*, 2005), and high levels of iron which are associated with free radical injury (Herbert *et al.*, 1994). Despite these "risk" factors for free radical generation and damage it is evident that antioxidant resources in the brain may be in fact lower than those found in other organs, for example catalase activity in the brain has been shown to be only 10% of that found in liver (Floyd & Carney, 1992).

The production of ROS/RNS can lead to cell injury and death through various mechanisms. Oxidative damage can cause cell membrane lipid destruction and cleavage of DNA (Vincent & Maiese, 2000; Wang *et al.*, 2003), peroxidation of cellular membrane lipids (Siu & To, 2002), peroxidation of docosahexaenoic acid which is a precursor of neuroprotective docosanoids (Mukherjee *et al.*, 2004), the cleavage of DNA during the hydroxylation of guanine and methylation of cytosine (Lee *et al.*, 2002), and the oxidation of proteins that yield protein carbonyl derivatives and nitrotyrosine (Adams *et al.*, 2001). ROS/RNS can also have a detrimental effect on mitochondria and are able to inhibit respiratory chain complex enzymes in the electron transport chain (**Figure 1.5**.)

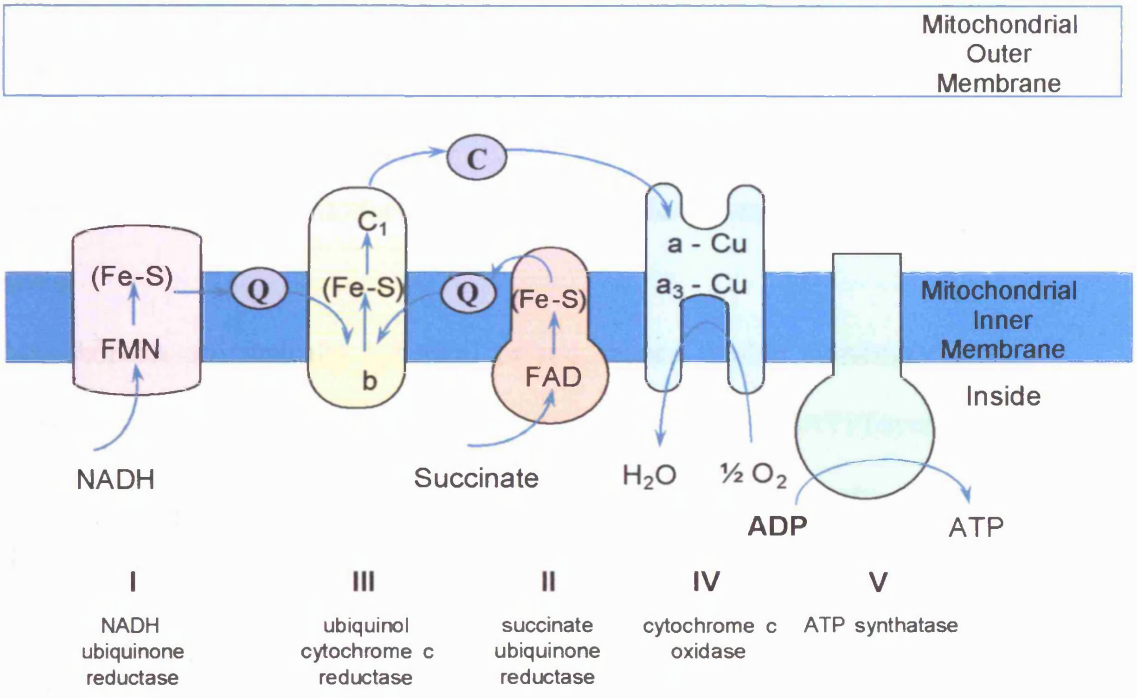


Figure 1.5. The mitochondrial respiratory chain.

The mitochondrial respiratory chain is an assembly of electron carriers grouped into four polypeptide complexes [I (NADH ubiquinone reductase), II (succinate ubiquinone reductase), III (ubiquinol cytochrome c reductase), and IV (cytochrome c oxidase). Complexes I, III, and IV act as oxidation-reduction-driven proton pumps and are associated with the coupling sites of phosphorylation. The flow of electrons from NADH or $FADH_2$, along the respiratory chain to oxygen is coupled to the pumping of protons across the inner mitochondrial membrane, resulting in the formation of a proton gradient. The flow of protons back into the mitochondria through the membrane sector of the mitochondrial ATP synthatase alters the active site of the enzyme leading to the synthesis of ATP.

Q = ubiquinone, C = cytochrome c both act as mobile electron carriers.

(Bolanos *et al.*, 1994, Yamamoto *et al.*, 2002) , resulting in energy failure and ultimately, cell death.

1.4.3. Mitochondria, the electron transport chain and sources of oxidative stress

Mitochondria are typically spherical or rod shaped double membrane bound organelles which are the major site of adenosine triphosphate (ATP) synthesis in the cell via oxidative phosphorylation. The inner membrane is the site of the mitochondrial electron transport chain (ETC), and where oxidative phosphorylation takes place (Mitchell, 1961). The ETC is comprised of more than 80 polypeptides which are grouped together into four enzyme complexes I - IV (Figure 1.5.) (Michel, 1998; Zhang *et al.*, 1998; Sazanov *et al.*, 2000). The ETC facilitates the transfer of electrons from NADH and $FADH_2$ which are generated by carbohydrate and fatty acid metabolism, to oxygen which is then reduced to water at complex IV (cytochrome oxidase, EC 1.9.3.1) (Michel *et al.*, 1998). Electrons are passed along the chain through the redox centres of the respiratory complexes, via increasing reduction potentials. Mobile electron carriers act to pass electrons from complexes I (NADH ubiquinone reductase, EC 1.6.5.3) and II (succinate ubiquinone reductase, EC 1.3.5.1) to complex III (Ubiquinol cytochrome c reductase, EC 1.10.2.2) (Ubiquinol) and from complex III to complex IV (cytochrome c) (Crofts *et al.*, 1999; Tormo & Estornell, 2000). Electron transport through the complexes is coupled to the pumping of protons from the matrix into the inter-membrane space by complexes I, III, and IV (Mitchell, 1961; Videira, 1998; Michel, 1998; Crofts *et al.*, 1999). This results in

an electrochemical gradient being formed across the membrane which is dissipated through the membrane domain of ATP synthase (EC 3.6.3.14) which leads to the phosphorylation of adenosine diphosphate (ADP) (Mitchell, 1961). The intramitochondrial compartment is highly reducing and various respiratory components including flavoproteins, iron- sulphur clusters and ubisemiquinone, are thermodynamically capable of transferring one electron to oxygen forming superoxide. (reviewed in Turrens, 2003). There are a number of mitochondrial sources of $O_2^{\bullet-}$ including several respiratory complexes and individual enzymes. Production of $O_2^{\bullet-}$ in mitochondria seems to vary from organ to organ and depends on whether mitochondria are actively respiring (state 3 respiration) or whether the respiratory chain is highly reduced (state 4 respiration) (Barja, 1999). It has been reported that complex III seems to be responsible for the majority of the $O_2^{\bullet-}$ produced in heart and lung mitochondria (Turrens and Boveris, 1980; Turrens *et al.*, 1982) but that in the brain it is complex I that is mainly responsible for $O_2^{\bullet-}$ production . Indeed in Parkinsons disease where oxidative stress is implicated as a causative factor complex I has been found to be the primary source of ROS (Betarbet *et al.*, 2002; Nicholls , 2002). Formation of $O_2^{\bullet-}$ is also likely to increase if the ETC begins to become reduced (State 4 respiration mentioned previously) by downstream "carriers" being inhibited causing electrons to "back-up" (Scheffler, 1999; Turrens, 2003).

1.6. HO and the brain

HO activity in the brain exceeds that of many systemic organs including the liver (Maines, 1997), This activity is mainly due to the action of the HO-2 isoform. Under normal unstressed conditions HO-2 is mainly expressed in neuronal

populations (Ewing & Maines,1992), and in the endothelial lining of blood vessels (Maines, 2000). HO-1 under normal unstressed conditions is only detectable in select neurons such as those in the dentate gyrus, cerebellum and brain stem (Sun *et al.*, 1990; Ewing & Maines, 1991; Ewing & Maines, 1992; Vincent *et al.*, 1994; Maines *et al.*, 1998). However, in response to stress, HO-1 is widely expressed in glial populations and macrophages (Ewing & Maines, 1991; Ewing & Maines, 1992; Ewing *et al.*,1992; Geddes *et al.*, 1996; Turner *et al.*, 1998).

Because of the differences in distribution and regulation of HO-1 and HO-2 in brain, it has been suggested that HO-2 is responsible for normal functioning, maturation, aging and homeostasis of gaseous signal molecules (Maines, 2000). HO-1, therefore, could be important in cellular defence mechanisms, as it has a rapid response to stressful stimuli. Due to the relative deficiency of glutathione and ascorbic acid in neuronal tissue (Ewing & Maines, 1993) the antioxidant molecules that HO-1 activity produces may be of key importance here (Ewing & Maines, 1993).

1.7. HO-1 and neurodegeneration

As mentioned previously, upregulation of HO-1 is seen in many disease states, whether or not they are involved in haem catabolism. Neurodegenerative diseases are not exempt from this. The most common of these diseases and possible roles for HO-1 in the disease process will be described

1.7.1. Aging

Although aging itself is a normal process and not a disease, neurodegeneration is a natural consequence of it. As previously mentioned HO-1 expression in normal brain is much less than that of HO-2 which under basal conditions is the main isoform of HO expressed in the brain. A study has recently shown that the numbers of neurons and neuroglia immunoreactive for HO-1 in the normal human cerebral cortex and hippocampus increase progressively between 3 and 84 years of age (Hirose *et al.*, 2003). It has also been shown that in normal elderly patients moderate HO-1 immunoreactivity was consistently observed in the cytoplasm of dopaminergic neurons of the substantia nigra pars compacta (Schipper *et al.*, 1998) . This is in contrast to staining of other neurons in this area, which was faint or nonexistent. This has been suggested to be a response to oxidative stress due to the high levels of ROS generated in these cells by the enzymatic deamination and iron-mediated oxidation of dopamine (Stokes *et al.*, 1999) which is a possible reason for age related depletion of nigral dopaminergic neurons at a faster rate compared to other brain neuron populations (Schipper 2004). Thus it is suggested that HO-1 expression in aging brain is indicative of an increased and ongoing oxidative stress.

1.7.1. Alzheimers disease

Alzheimers disease (AD) is an aging related dementia which features progressive neuronal degeneration, gliosis, and the accumulation of intracellular neurofibrillary tangles (NFT) and extracellular deposits of fibrillar amyloid (senile plaques) in nuclei of the basal forebrain, hippocampus and associated cortices (Selkoe, 1991). Implicated in the pathogenesis of this disease are

increased deposition of redox-active iron (Sayre *et al.*, 2001), chronic oxidative stress and mitochondrial dysfunction (Schipper, 2004; Casley *et al.*, 2002; Mattson, 2002).

Increased HO-1 expression has been observed in proven sporadic AD patient brains compared to non-demented control (Schipper *et al.*, 1995). Co-immunostaining for HO-1 and tau-2 has revealed colocalisation of HO-1 and the NFT associated with the AD brain (Smith *et al.*, 1994) and senile plaques from both AD and control brain were also noted to be immunopositive for HO-1 and β-amyloid, this was in plaques (Schipper *et al.*, 1995). Reasons for this pronounced upregulation of HO-1 in this condition are generally thought to be an antioxidative defense mechanism, one study has suggested that HO-1/tau interactions may prevent pathological hyperphosphorylation of tau and NFT formation (Takeda *et al.*, 2000). However it has also been shown that in rat astroglia, upregulation of HO-1 activity resulting from exposure to exogenous amyloid peptide facilitates the sequestration of iron by the mitochondrial compartment (Ham & Schipper, 2000). Thus continued overexpression of HO-1 could result in abnormal cerebral iron mobilization and mitochondrial damage, both of which have been associated with AD as already mentioned. Excessive HO-1 derived CO could also have a part to play in the pathogenesis of AD in that it could contribute to development of cognitive (Liebson & Albert, 1994), olfactory (Doty, 1991) and neuroendocrine (Sapolsky, 1992) disturbances characteristic of AD by increasing cyclic guanosine monophosphate in olfactory neurons (Verma *et al.*, 1993), affecting the secretion of corticotropin-releasing hormone (Parkes *et al.*, 1994), perturbing central vaso-regulatory mechanisms

(Marks *et al.*, 1991) and modulating hippocampal long term potentiation (Stevens & Wang, 1993; Zhou *et al.*, 1993).

1.7.3. Parkinsons disease

Parkinsons disease is a movement disorder with an onset usually after 65 years of age. Symptoms include resting tremor, rigidity, hypokinesia and postural instability (Schipper 2004). The disease is characterised by progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta and formation of α-synuclein-containing fibrillar inclusions (Lewy bodies) within this cell population. (Schipper 2004). Abnormally high levels of tissue iron have been reported in substantia nigra and basal ganglia of PD patients, and this may serve to generate ROS in this disease by reducing H_2O_2 to the hydroxyl radical and behaving as a nonenzymatic peroxidase activity capable of oxidizing dopamine and other catechols to toxic semiquinone intermediates (Riederer *et al.*, 1989; Jellinger *et al*, 1990; Schipper *et al.*, 1991; Morris & Edwardson, 1994; Jenner & Olanow, 1998). Dysfunction of dopaminergic neurons is a major focus in studies of PD, but it is suggested that activation of glial cells may play an important role in the initiation and progression of cell death (Hirsch *et al.*, 1998; McNaught & Jenner, 2000a; McNaught & Jenner, 2000b). Indeed it has been shown that excessive nigral iron seems to be predominantly deposited within astrocytes, migcroglia, macrophages and microvessels (Schipper, 2004). Upregulation of HO-1 expression has been well documented in PD. Cytoplasmic Lewy bodies from PD specimens have been shown to exhibit prominent HO-1 immunoreactivity (Castellani *et al.*, 1996; Schipper, 2004) , expression of HO-1 in the neuropil of the PD nigra was generally more pronounced than in control

counterparts (Schipper *et al.*, 1998) and *in vitro* physiological concentrations of dopamine (1μM) used to treat astroglial cultures rapidly upregulated HO-1 gene transcription and enzyme activity causing mitochondrial oxidative stress and permeabilistion of the mitochondria to nontransferrin iron and other transition metals (Schipper , 2004). It is suggested that pathological expression of HO-1 in astrocytes within the PD nigra may provide an explanation for the perturbed iron homeostasis and oxidative mitochondrial damage observed in affected tissues (Beal, 2003).

1.7.4. Multiple Sclerosis

Multiple sclerosis (MS) is a disease of the central nervous system characterised by dysregulation of cytokine production, inflammation, oligodendroglial degeneration , multifocal demyelination, axonal degeneration and astrogliosis (Schipper, 2004). There is evidence that aberrant iron deposition and oxidative stress have a role to play in the pathogenesis of this disease (Levine & Chakrabarty, 2004). GFAP positive astrocytes expressing HO-1 in spinal cord plaques from MS patients were considerably greater than that in the spinal white matter of normal subjects (Mehindate *et al.*, 2001) and proinflammatory cytokines secreted in MS-affected tissues promoted sequestration of non-transferrin derived ^{55}Fe by astroglial microglia, which could be prevented by co-administration of HO inhibitors, mitochondrial permeability transition pore blockers or antioxidants (Mehindate *et al*, 2001). It is suggested that overexpression of HO-1, caused by inflammatory cytokines (Mehindate *et al.*, 2001) or myelin basic protein (Businaro *et al.*, 2002) could contribute to

inappropriate pathological iron mobilisation and electron transport chain defects seen in the vicinity of MS plaques (Lu *et al.*, 2000).

1.8. Inducible nitric oxide synthase, NO and neurodegeneration

As discussed in the previous section HO-1 expression is associated with many disease states. In the neurodegenerative diseases mentioned (at least) another common feature is pathological production of NO via activation of inducible nitric oxide synthase (iNOS). Physiologically NO is generated by NO synthase (NOS; EC 1.14.13.39). There are three known isoforms of this enzyme, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (for review see Alderton *et al.*, 2001). These enzymes catalyse, in the presence of molecular oxygen, tetrahydrobiopterin and other cofactors, the conversion of arginine to NO plus citrulline (Lincoln *et al.*, 1997). NO has been ascribed a number of important biochemical roles within the central nervous system including cGMP formation following the activation of soluble guanylyl cyclase and the regulation of the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase (Garthwaite *et al.*, 1988; Heales *et al.*, 1997). However NO is a free radical and in many biological systems has a short half life due to its reactivity with other intracellular constituents, such as superoxide (Beckman *et al.*, 1993), this reaction forms the reactive nitrogen species peroxynitrite ($ONOO^-$) which is cytotoxic (Lipton *et al.*, 1993) as discussed previously. Excessive production of NO and its ability to form $ONOO^-$ have been implicated in the pathophysiology of a number of neurodegenerative diseases, where mitochondrial damage is a factor, including PD, AD, MS, ischaemia and amylotrophic lateral sclerosis (Heales *et al.*, 1999; Stewart & Heales, 2003).

NO-mediated inhibition of complex I has been demonstrated in some studies (Stadler *et al.*, 1991; Geng *et al.*, 1992) although it has been reported that this inhibition is due to $ONOO^-$ rather than NO (Cassina & Radi, 1996; Riobo *et al.*, 2001). However, other groups have reported that complex I is relatively resistant to $NO/ONOO^-$ mediated inhibition (Bolanos *et al.*, 1994; Bolanos *et al.*, 1995; Stewart *et al.*, 1997). A number of factors may be responsible for this apparent discrepancy of results; concentration of mitochondria used, respiratory substrates used, GSH status of cells or mitochondria used or type of NO donor (Stewart & Heales, 2003).

The activities of complex II and complex III are often assayed together using a single assay (King, 1967), and mitochondrial complex II + III has been reported to be vulnerable to $NO/ONOO^-$ exposure (Bolanos *et al.*, 1994; Bolanos *et al.*, 1995, Stewart *et al.*, 1997).

NO is able to reversibly and irreversibly inhibit complex IV (Keilin & Hartree 1939). NO reversibly inhibits by binding to complex IV in competition with oxygen (Stevens *et al.*, 1979). Irreversible inhibition has been reported following prolonged NO exposure of mitochondria (Poderoso *et al.*, 1996) and cells (Bolanos *et al.*, 1996). It is reported that exposure of astrocytes to cytokines which may be elevated in inflammatory neurodegenerative disease, for example IFNγ causes generation of reactive nitrogen species, leading to inhibition of the mitochondrial respiratory chain (Bolanos *et al.*, 1994).

1.9. Interactions between the HO and iNOS enzyme systems

There are obvious similarities between the HO and NOS systems. Both have inducible and constitutive forms. The inducible form of NOS is is responsive to

certain of the stimuli which induce HO-1 (Maines, 1997), although HO-1 is known to have many more inducers. Both systems produce gases that have important physiological functions, and both these gases by their affinity for the haem molecule are able to bind soluble guanylyl cyclase and cause it to activate cGMP (see review, Alderton *et al.*, 2001). Both of the constitutive forms are regulated by the adrenal glucocorticoids, although in opposite ways, expression of HO being upregulated and NOS downregulated (Maines, 1997).

It is recognized that these two enzyme systems have regulatory effects on each other, with many reports that NO upregulates HO-1 production (Bouton & Demple, 2000, Datta & Lianos, 1999, Foresti *et al.*,1997) though interestingly Willis *et al.*, (1995) report that the NO donor sodium nitroprusside (SNP) reduced HO activity in both brain and spleen. HO can modulate NOS activity and therefore production of NO in a number of ways. Increased HO-1 activity caused by NO production could degrade the haem located in the active site of NOS or deplete available haem for de novo synthesis of NOS (Maines, 1997). Iron released by HO action could further inhibit NOS by inhibiting its nuclear transcription. Also both enzymes require NADPH as a co-factor, and the subsequent reduction of biliverdin by biliverdin reductase also utilizes NADPH, which could shift competition for electrons in favour of the HO pathway (Maines, 1997). NOS activity can also be directly affected by inhibition by CO binding to the haem active site. Studies have shown that exogenous CO inhibits both macrophage and rat cerebellar NOS activity (White & Marletta, 1992). It has been shown though, that the effect that CO has can depend on the amount of CO in the system. Thorup *et al.*, (1999) report that high levels of CO inhibit NOS

activity and NO generation whereas lower concentrations induce release of NO from intracellular pools.

The links between the HO and NOS enzyme systems lead to questions about the role that HO may play in neurodegeneration. As mentioned previously, CO binds to cytochrome *c* oxidase (Piantadosi, 2002) due to its affinity for the haem moiety. Thus CO, like NO, can cause increased electron leakage and hence formation of the superoxide anion, leading to oxidative stress, mitochondrial inhibition and ATP depletion. It has been documented that CO poisoning can cause oxidative stress (Piantadosi *et al.*, 1997; Thom, 1990; Thom *et al.*, 1997; Zhang & Piantadosi, 1992). Mitochondrial damage caused by NO has been implicated in various neurodegenerative disorders (Heales *et al.*, 1999). In these disorders upregulation of HO-1 is also reported (Schipper, 2000), thus leading to a possible rise in endogenous CO levels. In this case could it be possible that HO derived CO is responsible for a proportion of the mitochondrial damage previously attributed to NO? We know that the capacity of the brain to generate CO may exceed its capacity to generate NO (Maines, 1993). Moreover, Thom *et al.*, (1997) report that CO competes for intracellular sites that normally bind NO, which increases the steady state level of unbound NO available to undergo reactions with superoxide. So CO may be likely to potentiate oxidative stress by binding to cytochrome *c* oxidase and also make available NO, thus increasing the possibility of nitrosative stress.

1.10.The aims of the thesis

In view of the suggestion that HO-1 induction can act in both a beneficial and a deleterious way within the cell and the fact that upregulation of HO-1 is reported in many neurodegenerative diseases, this thesis has examined the effects of HO induction in rat astrocytes and interactions with the NO/iNOS system. As previously discussed induction of iNOS in rat astrocytes with IFNγ/LPS causes inhibition of complex IV of the ETC; since it has already been shown that IFNγ/LPS also induce HO-1 expression (Kitamura et al., 1998) this thesis will examine whether this expression is accompanied by enzyme activity, and if so what is the consequence for the cell if this activity is augmented or inhibited. The thesis will be set out as follows:

1) Establishment and validation of an assay to determine HO activity in rat astrocytes.

2) Induce HO-1 activity and expression in rat astrocytes and examine the effects on the ETC, GSH and nitrite/nitrate status

3) Induction of iNOS activity and examination of the effects of this on HO-1 expression and HO activity

4) Investigation of the consequences of manipulating the HO-1 status of iNOS activated astrocytes and the effects this has on NO- mediated respiratory chain inhibition, HO activity and GSH status.

Chapter 2.

General Materials and Methods

2.1 Materials.

Unless otherwise stated all chemicals were of analytical grade and purchased from Sigma-Aldrich Company Ltd (Poole, UK), or VWR International Ltd (Leicester, UK). In particular:

Deoxyribonuclease 1 (from bovine pancreas, EC 3.1.21.1), Earle's Balanced Salt Solution, Hank's Balanced Salt Solution, bovine serum albumin, L-glutamine, antibiotic antimycotic solution (100x), trypsin-EDTA solution (10x, porcine trypsin), rotenone, coenzyme Q_1, antimycin A and L-N6-(L-iminoethyl) lysine hydrochloride (L-NIL) were purchased from Sigma-Aldrich Company Ltd (Poole, UK).

Tissue culture plastics and spectophotometric grade chloroform were purchased from VWR International Ltd (Leicester, UK).

Trypsin (from bovine pancreas, EC 3.4.21.4), oxidised cytochrome c (from horse heart) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were purchased from Roche Diagnostics (Lewes, UK).

Minimal essential medium and foetal bovine serum were purchased from Invitrogen, (Paisley, UK).

Hemin was purchased from Frontier Scientific Europe Ltd (Lancashire, UK).

Tin protoporphyrin IX (SnPPIX) and zinc protoporphyrin IX (ZnPPIX) were purchased from Affiniti Research Products Ltd (Exeter, UK).

Sephadex G-25M (PD-10 columns; 3.5ml elution) used to remove ascorbate from reduced cytochrome c were bought from Amersham Biosciences (Little Chalfont, UK).

The nitric oxide (NO) donor, (Z)-1-[2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) was purchased from Alexis Biochemicals (Nottingham, UK)

Antibodies to haem oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS) were purchased from Bioquote Ltd (York, UK).

The mouse monoclonal anti-glial fibrillary acidic protein was purchased from Sternberger Monoclonals Inc. (Lutherville, Maryland, USA).

The Bio-Rad protein assay kit and 12% TRIS-HCl mini prep gels were purchased from Bio-Rad Laboratories (Hercules, California, USA)

Interferon gamma (IFNγ) was purchased from CN Biosciences (California, USA).

2.2. Tissue culture

2.2.1. Cell Culture Media Composition

Solution A was composed of Earle's Balanced Salt Solution (EBSS) containing deoxyribonuclease I (75 KU/ml), 1% vol/vol antibiotic antimycotic solution (10 units/ml penicillin, 1μg/ml streptomycin and 2.5ng/ml amphotericin B) and 3mg/ml bovine serum albumin (BSA).

Solution B was composed of solution A supplemented with 112.5 KU/ml deoxyribonuclease I and 27.5 units/ml trypsin.

Astrocyte medium was composed of L-valine based minimal essential media (MEM) supplemented with 2mM L-glutamine , 10% (v/v) foetal bovine serum and 1% (v/v) antibiotic antimycotic solution.

2.2.2. Animals

Pregnant Wistar rats were purchased from A.J. Tuck and Sons Ltd. (Rayleigh, Essex, U.K.), fed ad libitum on a stock laboratory diet, and were maintained on a 12 hour light dark cycle.

2.2.3. Primary astrocyte culture

Primary cortical rat astrocytes were isolated from neonatal (0-2 days) Wistar rats by a method adapted from Tabernero *et al* (1993). Neonates were decapitated and the cortices removed. Each cortex was then held with a pair of dissecting tweezers and "rolled" on filter paper (Whatman Type 1) to remove blood and meninges and prevent the growth of fibroblasts. The cortices were then triturated in solution A (section 2.2.1). The triturated brain solution was then centrifuged at 500 x g for 5 minutes at 4°C. The supernatant was discarded and the pellet enzymatically digested in solution B (section 2.2.1) for 15 minutes at 37°C. Digestion was terminated by adding 1ml foetal bovine serum, and the astrocytes were pelleted by centrifugation at 500 x g for 5 minutes at 4°C. The resulting pellet was resuspended in 10ml of solution A and debris was allowed to settle out for ≈ 5 minutes. The supernatant was carefully removed with a 1ml Gilson pipette and centrifuged at 500 x g for 5 minutes at 4°C. The pellet was resuspended in astrocytic medium (section 2.2.1) and passed through nylon gauze (100μM pore size) to remove any remaining cell debris. Astrocytes were plated in 80-cm^2 flasks (2 heads per flask) and cultured in astrocytic medium in a Heraeus incubator (95% air/5% CO2) at 37°C, and medium was changed every third day.

2.2.3.1 Passage of astrocytes

Astrocytes were passaged on day 7 when they reached confluence. Cell media was removed and cells washed with Hank's Balanced Salt Solution (HBSS), and

incubated with 10ml trypsin/EDTA solution (0.5% (w/v) trypsin, 0.2% (wt/vol) EDTA) for 5 minutes. Trypsinisation was terminated by the addition of 5% (v/v) foetal bovine serum, and the astrocytes pelleted by centrifugation at 500 x g for 5 minutes at 4°C. Astrocytes were then resuspended in astrocytic medium (to a volume twice that before trypsinisation) and cultured in flasks for a further 6 days as described previously.

2.2.3.2 Plating and treating of astrocytes

Astrocytes (Figure 2.1.) that were to be treated in 6-well plates were removed from the flasks on day 13 as described above, (section **2.2.3.1**.) and resuspended in astrocyte media. The cells were counted and then seeded onto poly-l-lysine coated (10μg/ml) plates at a density of 1.04×10^5 cells/cm^2. The cells were then incubated for 18-24 hours to allow them to attach. For treatment, media was removed and replaced with fresh media containing specified treatments for defined periods of time. For the HO assay cells were treated in flasks. On day 13 media was removed and replaced with fresh media containing specified treatments for defined periods of time.

2.2.3.3 Harvesting of cells

Following treatment in 6-well plates, cells were harvested by trypsinisation. Media was removed and cells washed with 1ml HBSS. 1ml trypsin/EDTA solution was added and incubated at room temperature for 2 minutes. Trypsinisation was terminated by the addition of 100μl foetal calf serum (FCS).

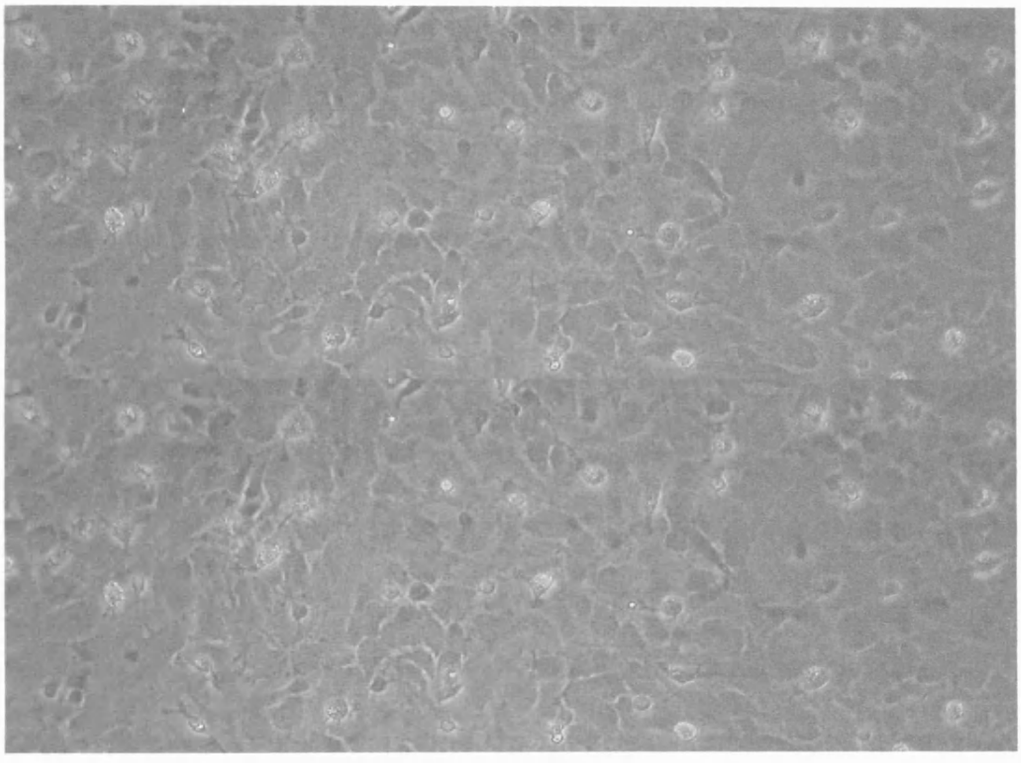


Figure 2.1. Phase contrast image of confluent primary rat astrocytes at day 14 of culture. 10x objective.

Cells were collected and centrifuged at 500 x g for 5 minutes at 4°C. Pellets were then resuspended in appropriate buffer for assay and if necessary snap frozen in liquid nitrogen and stored at -70°C until assay. For HO assay, media was removed from flasks and cells washed with 10ml HBSS. Cells were then scraped using a long handled cell scraper. Cells and HBSS were collected and centrifuged at 500 x g for 5 minutes at 4°C. Pellets were resuspended in 500µl HO buffer (2mM $MgCl_2$, 100mM K_2HPO_4, 100mM KH_2PO_4 pH 7.4), frozen in liquid nitrogen and stored at -70°C until assay.

2.2.3.4. Immunostaining of astrocytes

To determine the astrocytic content of cultures, staining for glial fibrillary acidic protein (GFAP) was used. GFAP is a well-established marker for astrocytes as it is the principal intermediate filament of mature astrocytes in the central nervous system (Eng *et al.*, 2000). Cells were seeded at a density of approximately 1.3 x 10^3 cells/cm^2 onto 25mm glass cover slips pretreated with poly-l-lysine, and allowed to attach for 24 hours. After this time the astrocyte media was removed and the cells were washed 3 times with phosphate buffered saline (PBS; 0.14M NaCl, 2mM KH_2PO_4, pH 7.4). Astrocytes were then fixed by addition of 1ml ice-cold (-20°C) methanol per well for 5 minutes. The methanol was removed and the cells washed with PBS three times. Each cover slip was then incubated in 1ml 1%(v/v) horse serum for 30 minutes (room temperature) in order to prevent non-specific binding before being washed three times with PBS once more. 1ml of mouse derived primary antibody to GFAP (1:2000 dilution in PBS) was then added to the coverslips before overnight incubation at 4°C. One coverslip was

incubated with PBS only as a blank control. After incubation the coverslips were washed three times with PBS , and then incubated with 1ml of secondary antibody (fluorescein isothiocyanate (FITC)-conjugated secondary anti-mouse immunoglobulin-G antibody (1:500 dilution in PBS) for 1 hour at room temperature. Non-specific binding of the secondary antibody was determined by incubating it with astrocytes which had not been exposed to the primary antibody. Once more coverslips were washed three times with PBS and then incubated with 1ml 0.01% 4'-6-diamidino-2-phenylindole (DAPI) in the dark for 10 minutes in order to stain cell nuclei and ascertain cell numbers. The coverslips were washed 3 times finally and mounted on glass microscope slides (BDH Ltd; 76 x 26 mm) using 10µl Cytoflour (Cytoflour Ltd. London, UK).

Digital imaging of cells was performed using a Zeiss 510 CLSM laser scanning confocal microscope (Solent Scientific, Portsmouth, UK) equipped with a 40x oil immersion quartz objective lens and a 20x quartz objective lens. The fluorescence of the FITC-conjugated antibodies was imaged by illuminating cells using the 488 nm emission line of a helium-neon laser, and the fluorescence signal was collected at wavelengths longer than 505 nm. In order to image DAPI fluorescence, cells were illuminated using the 351 nm laser line of an argon laser and the fluorescence signal was collected at 435 and 485 nm. The microscope pinhole was maintained at a confocal thickness of about 5mm.

The purity of the cultures was calculated by determining the proportion of DAPI fluorescence containing cells that were also positive for GFAP fluorescence. Astrocyte cultures showed a 94 ± 2% immunopositivity against GFAP (**Figure**

2.2.). No GFAP fluorescence was detected in the blank control incubated with the secondary antibody alone.

2.3.Enzyme assays

2.3.1 Complex I Assay (NADH:ubiquinone reductase; EC 1.6.5.3)

Complex I activity was determined as described by Ragan *et al.*, (1987) with modifications. Samples (10-20μg), suspended in sucrose-Hepes buffer (0.25M sucrose, 2 mM Hepes pH 7.2) were freeze-thawed three times in liquid nitrogen and mixed with assay buffer (8mM $MgCl_2$, 20mM KH_2PO_4, 20mM K_2HPO_4, pH 7.2), 2.5mg/ml BSA (fatty acid free), 1mM KCN and 0.15mM NADH, in 1ml cuvettes. The reaction mixture was incubated at 30°C for 2 minutes prior to the addition of 50μM coenzyme Q_1. Enzyme activity was measured at 30°C by following the oxidation of NADH to NAD^+ for 5 minutes (NADH extinction coefficient 6.81 x 10^3 $M^{-1}cm^{-1}$; total volume 1ml; path length 1cm) using an Uvikon 941 spectrophotometer. Each sample was measured against a blank which contained sample and all substrates except coenzyme Q_1. After 5 minutes 20μM rotenone was added to the sample cuvettes which were measured for a further 5 minutes in order to measure rotenone insensitive NADH oxidation. Complex I activity was calculated by subtracting the rotenone insensitive NADH oxidation rate from total NADH oxidation rate (units = nmol/min/mg protein). Complex I activity was proportional to protein between 5 and 20μg protein (R^2 0.9833).

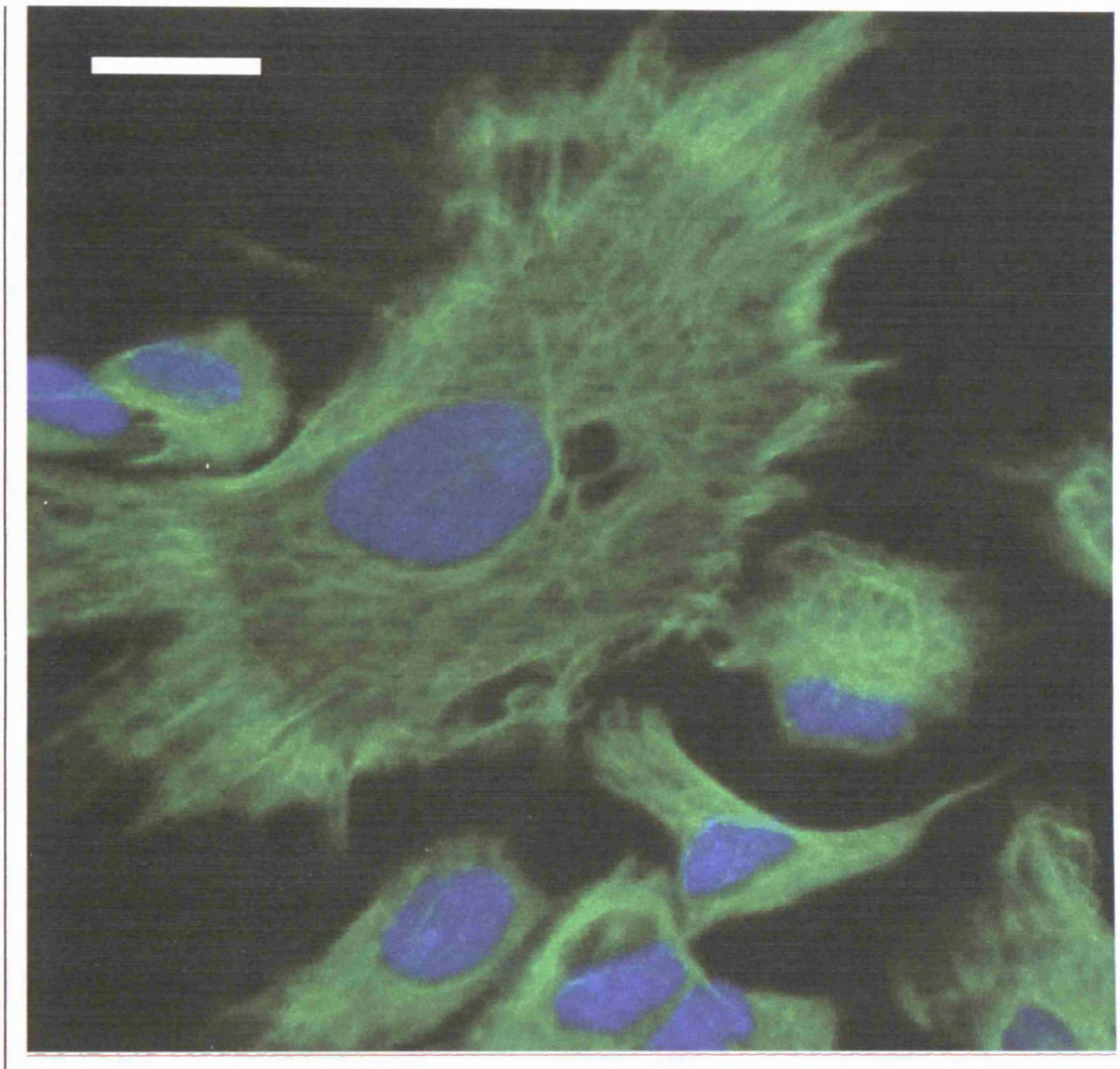


Figure 2.2. Image of glial fibrillary acidic protein positive astrocytes (green). Cell nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI) (blue) Bar = 10μM.

2.3.2. Complex II/III assay (succinate cytochrome c reductase; EC 1.8.1.3)

Complex II/III activity was determined in a linked assay as described by King (1967). Electrons are transferred through complex II by the oxidation of succinate , to ubiquinone (CoQ_1), this reduces CoQ_1 to ubiquinol. Complex III reduces cytochrome *c* by the transfer of electrons from ubiquinol, and this simultaneously oxidises ubiquinol back to CoQ_1. Activity therefore was measured by the reduction of cytochrome c at 550nm (cytochrome *c* extinction coefficient 19.2×10^3 $M^{-1}cm^{-1}$; total volume 1ml; path length 1cm) using a Uvikon 941 spectrophotometer. Samples (10-20mg protein; freeze- thawed 3 times in liquid nitrogen) were mixed with assay buffer (100mM KH_2PO_4, 100mM K_2HPO_4, pH 7.4), 0.3mM EDTA, 1mM KCN, and 100µM oxidised cytochrome c (from horse heart) in a cuvette. The reaction was started by the addition of 20mM succinate and enzyme activity was measured at 30°C for 5 minutes. At this time 10µM antimycin A was added and the antimycin A insensitive rate of cytochrome *c* reduction was measured for a further 5 minutes. Complex II/III activity was calculated by subtracting the antimycin A insensitive cytochrome c reduction rate from total cytochrome *c* reduction rate (units = nmol/min/mg protein). Each sample was measured against a blank which contained all substrates except succinate. Complex II/III activity was proportional to protein between 5 and 25µg protein (R^2 0.9833).

2.3.3. Complex IV assay (cytochrome c oxidase; EC 1.9.3.1)

2.3.3.1. Reduction of oxidised cytochrome c

Ascorbate crystals were added to oxidised cytochome *c* (0.8mM from horse heart) and a colour change was observed from dark red to light red. The ascorbate was removed from the reduced cytochrome *c* by passing it through a PD10 gel filtration column (column equilibrated by first washing with ≈ 50ml of 10mM phosphate buffer; 10mM KH_2PO_4, 10mM K_2HPO_4 pH 7.0) Concentration of reduced cytochrome c was determined by mixing 50µl reduced cytochrome *c* with 950µl H_2O in sample and reference cuvette. The cuvettes were "blanked" against each other at 550nm and 1mM potassium ferricyanide was added to the reference cuvette to re-oxidise the cytochrome *c*, and the absorbance of the sample cuvette noted (cytochrome c extinction coefficient $19.2x10^3M^{-1}cm^{-1}$; total volume 1ml; path length 1cm).

2.3.3.2. Measurement of complex IV activity

Complex IV activity was determined following the method of Wharton & Tzagoloff (1967). In a sample and reference cuvette, assay buffer (10mM KH_2PO_4, 10mM K_2HPO_4, pH 7.0) and 50µM reduced cytochrome *c* were mixed, and the sample cuvette "blanked" against the reference. 1mM potassium ferricyanide was added to the reference cuvette, to oxidise the cytochrome *c*. The reaction was started by adding sample to the sample cuvette (10-20µg protein; freeze-thawed three times in liquid nitrogen). The oxidation of cytochrome *c* was

followed for 5 minutes at 30°C using a Uvikon 941 spectrophotometer (cytochrome c extinxtion coefficient $19.2x10^3$ $M^{-1}cm^{-1}$; total volume 1ml; path length 1cm). Since complex IV activity is dependent on the concentration of cytochrome *c*, complex IV activity is expressed as the first order rate constant k per minute per mg of protein. Activity was determined by noting the highest positive absorbance following sample addition (t = 0 minutes), and the absorbance every minute after that for 3 minutes. *k* was calculated by: ((ln ($A_{550\ t=0}$/ $A_{550\ t=n}$) / number of minutes)/ protein concentration. The rate constant for each sample was taken as the mean of *k* at 1,2 and 3 minutes. Complex IV activity was proportional to protein between 15 and 30µg of protein (R^2 0.9905).

2.3.4. Citrate Synthase Assay (EC 4.1.3.7)

Citrate synthase (CS) activity was determined using a method described by Shepherd and Garland (1969). Sample (10-20 µg protein; freeze-thawed three times in liquid nitrogen) was mixed with buffer (100mM Tris, 0.1% (v/v) Triton X-100, pH 8.0), 0.1mM acetyl coenzyme A, and 0.2mM 5,5' dithio-bis-(nitrobenzoic acid)(DTNB) in a cuvette (total volume 1ml, path length 1cm). The reaction was started by the addition of 0.2mM oxaloacetate and activity measured using a Uvikon 941 spectrophotometer for 5 minutes. Activity was measured at 412nm and at 30°C (DTNB extinction coefficient $13.6x10^3$ $M^{-1}cm^{-1}$). Samples were run against a reference cuvette which contained sample and all substrates except oxaloacetate. Citrate synthase activity was linear with respect to protein between 5 and 25µg protein (R^2 0.9822).

2.3.5. Lactate Dehydrogenase Assay (EC 1.1.1.27)

Lactate dehydrogenase activity was determined spectrophotometrically as described by Vassault (1983). LDH released into 1ml of cell culture media during the treatment period, by $1x10^6$ astrocytes, was used as an index of cell viability. Assay buffer (100mM KH_2PO_4, 100mM K_2HPO_4, 170mM sodium pyruvate, pH 7.5) was mixed with 0.16mM NADH in the sample and reference cuvettes. Absorbance was then measured using a Uvikon 941 spectrophotometer for 5 minutes at 340nm after the sample and reference cuvettes had been "blanked" against each other. Absorbance was measured at 30°C (NADH extinction coefficient $6.81x10^3$ M^{-1} cm^{-1}; total volume 1ml; path length 1cm). The % release of LDH into culture medium was calculated as: LDH activity in media/ LDH activity in media + cells (Bolaños *et al.*, 1995).

2.4. Total Nitrate and Nitrite

In order to provide an indication of NO production in cells it is possible to measure the NO breakdown products, nitrite and nitrate using the Griess assay (Green *et al.*, 1982). Phenol red free media must be used in order that the phenol red does not interfere with the colourmetric reaction.

The Greiss assay is based on the reaction between nitrite and sulphanilamide in acidic solution which forms an intermediate diazonium salt that couples to N-(1-napthyl)ethylenediamine to yield a purple azo derivative that can be monitored by absorbance at 540nm. Standards of sodium nitrite and sodium nitrate

(0,10,20,30,40,50,100μm) were made up in phenol red-free medium. Another set of standards were made up in phenol red-free medium that contained 100μm hemin, in order that absorbance due to hemin could be taken into account.

50μl samples and standards were pipetted into 96 well plates in duplicate. Since the Griess reagents only measure nitrite it was then necessary to convert any nitrate present to nitrite in order to accurately measure all breakdown products. This was done using nitrate reductase (EC 1.6.6.2) (from Aspergillus, Roche Diagnostics, Lewes, UK). 10μl of each of the following reagents were added to each well (final concentrations): 5μM flavin adenine dinucleotide disodium salt (FAD), 100μM NADPH and 0.1units/ml nitrate reductase. The plate was then incubated at 37°C for 15 minutes in the dark. 10μl of each of the following reagents were then added to each well (final concentrations): 100 units/ml lactate dehydrogenase and 100mM sodium pyruvate in order to remove excess NADPH via oxidation to prevent it interfering with detection with the detection of nitrite by the Griess reagents (Medina A and Nicholas DJD (1957), in Green *et al.*, 1982). The plate was then incubated at 37°C for a further 5 minutes. The Griess reagents were prepared immediately before addition by combining 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride in water and 1% sulphanilamide in 5% (w/v) H_3PO_4. 100μl Griess reagent was added to each well and the plates were incubated in the dark for 15 minutes at room temperature. The absorbance of each well was read at 540nm using a spectrophotometric plate reader (SpectraMax Plus®, Molecular Devices, Wokingham, UK). The action of nitrate reductase was confirmed each time the assay was performed by using two standard curves to ensure that sodium nitrate had been reduced to sodium nitrite. **(Figure 2.3.)**

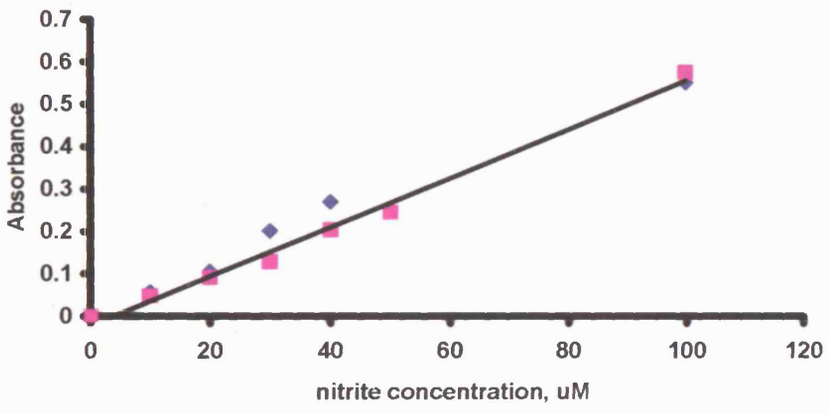


Figure 2.3. Greiss assay standard curve

The representative nitrite standard curve shows the absorbance of both nitrite standards (♦) and the reduced nitrate standards (■).

2.5. Western Blot analysis

Cells for western blot analysis were lysed on ice with lysis buffer (0.27M sucrose, 20mM Tris, 1mM EDTA, 1mM EGTA, 10mM Na β glycerophosphate, 1mM Na orthovanadate, 5% glycerol, 1% triton-x 100 pH 7.4) for 10 minutes , then sonicated for 30 seconds. Each sample was then centrifuged at 14000g for 10 minutes. The resulting supernatant was used for HO-1 or iNOS protein level determination. Equal amounts of protein (10μg) were loaded onto a 12% TRIS-HCL ready gel and separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (180V for 1 hour). The proteins were transferred to a nitrocellulose membrane using a semi-dry blotting system (100mA for 1 hour) (Figure 2.4.). The membrane was checked for protein transfer using Ponceau red. Unspecific binding sites on the membrane were blocked using 5% skimmed milk blocking buffer ((5% skimmed milk powder in Tris buffered saline – Tween 20 (20mM Tris base, 137mM NaCl, 0.05% Tween-20)pH 7.6)) (TBS-T) for 1 hour at room temperature. Membranes were then probed with polyclonal HO-1 or iNOS antibodies (Stressgen, Victoria, Canada) for 2 hours at room temperature (HO-1 1:4000, iNOS 1:5000, in TBS-T). The membrane was washed 3 x 10 minutes in TBS-T and then incubated with the secondary antibody, ECL Anti-rabbit IgG, peroxidase-linked species specific whole antibody from donkey (Amersham Biosciences UK Ltd, Buckinghamshire, UK) (1:4000 dilution).

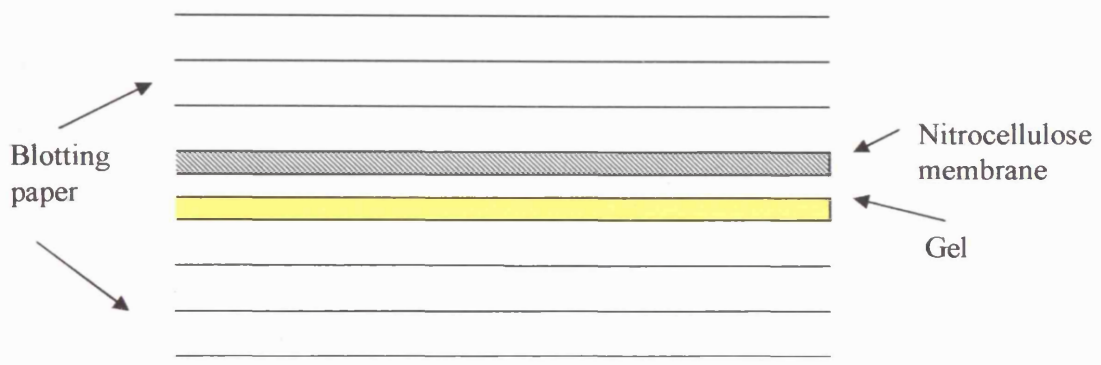


Figure 2.4. Semi-dry transfer of proteins after SDS-PAGE

Schematic to show the "transfer sandwich" for a semi-dry Western blot. Six sheets of blotting paper cut to size were soaked briefly in transfer buffer (48mM Tris/HCL, 39mM glycine, 0.0375% SDS, 20% MeOH, pH 9.2). Three sheets of blotting paper were assembled on top of each other on the Trans-Blot semi-dry transfer cell (Bio-Rad). The gel was soaked in transfer buffer and placed on top of the first three sheets of blotting paper. A piece of nitrocellulose membrane (Hybond ECL Nitrocellulose, Amersham) was cut to size, soaked in transfer buffer and placed carefully on top of the gel ensuring no air bubbles were created. The last three sheets of blotting paper were then placed on top of the membrane. The lid of the transfer cell was closed securely, compressing the "sandwich" and a current applied across the cell, 0.1A for 1 hour to effect protein transfer.

Protein-antibody complexes were detected using an ECL plus western blotting detection system (Amersham Biosciences UK Ltd. Equal protein loading was confirmed by probing the membrane with a β- actin antibody (Sigma). Molecular masses were determined by using Bio-Rad Precision Plus protein standards.

2.6. GSH Quantitation

2.6.1 Chromatography of reduced glutathione

Reduced glutathione (GSH) was measured using reverse phase HPLC with electrochemical detection (**Figure 2.5**) based on the method of Reiderer *et al.*, (1989). Sample (20µl) was injected by a Kontron HPLC 360 autosampler (Watford, U.K) through a guard column (octadecasilyl; 3mm x 10mm) to remove any debris, and resolved using a reverse-phase Techsphere octadecasilyl column (particle size 5µm, 4.6mm x 250mm) which was maintained at 30°C by a column heater (Jones Chromatography; Glamorgan, U.K.). Mobile phase was 15mM orthophosphoric acid prepared in de-ionised water that had been purified to electrical resistance of < 18 MΩ.cm-1 using a milli-Q apparatus. This was degassed by a DEG-1033 degasser (Kontron Instruments). Flow rate was maintained at 0.5ml/min by a Jasco PU-1580 pump (Great Dunmow, U.K).

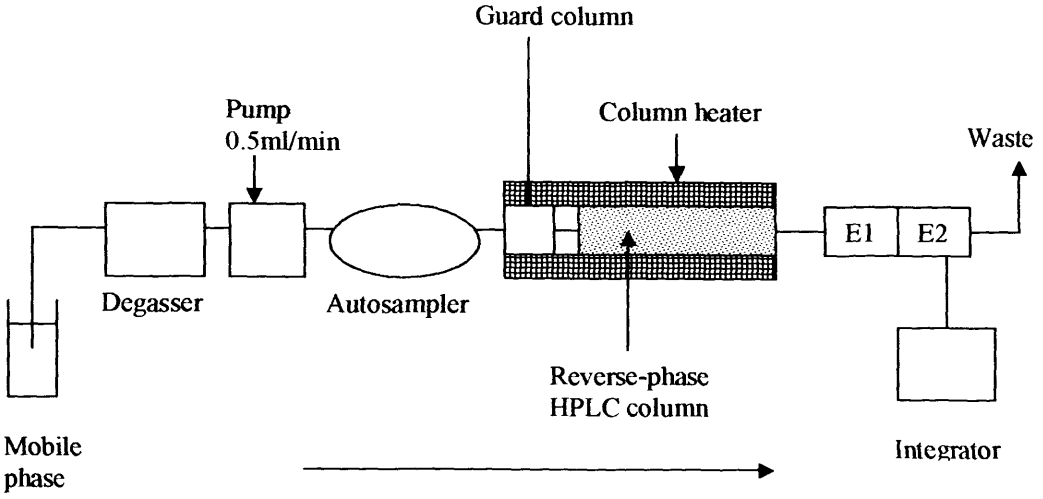


Figure 2.5. Scheme to illustrate the equipment used to measure GSH by reverse-phase HPLC and electrochemical detection.

E1 = upstream electrode; E2 = downstream electrode.

2.6.2. Electrochemical detection of GSH

Following separation by the column GSH was detected by an ESA 5010 analytical cell containing an upstream and downstream electrode (ESA Analytical; Aylesbury, UK). The upstream electrode was set at + 100mV to screen out molecules with a lower oxidation potential than GSH. In order to determine the optimal potential to detect GSH at the downstream electrode GSH standards (10μM) were injected and measured at various downstream electrode potentials (**Figure 2.6**.). GSH detection by the downstream electrode reached a plateau between 800mV and 850mV. The current generated by the oxidation of GSH was proportional to the amount of GSH, which was detected at the downstream electrode. This was recorded as a chromatogram on a Thermoseparation Products chromejet integrator (Anachem; Luton, U.K).

2.6.3. Sample preparation

GSH standards (1-10μM) were prepared in 15mM orthophosphoric acid and stored at -70°C. Samples resuspended in HBSS were diluted 1:1 (vol/vol) with 15mM orthophosphoric acid in order to extract GSH. Samples were then centrifuged for 5 minutes at room temperature at 14000 x g to pellet any protein. 200μl of supernatant was sealed in Chromacol HPLC ready for injection onto the HPLC column. A typical GSH chromatogram is shown in **Figure 2.7**. Results were expressed nmol/mg protein. Electrochemical detection of GSH standards was linear between 1 and 10μM (**Figure 2.8.** $R^2 = 0.99$).

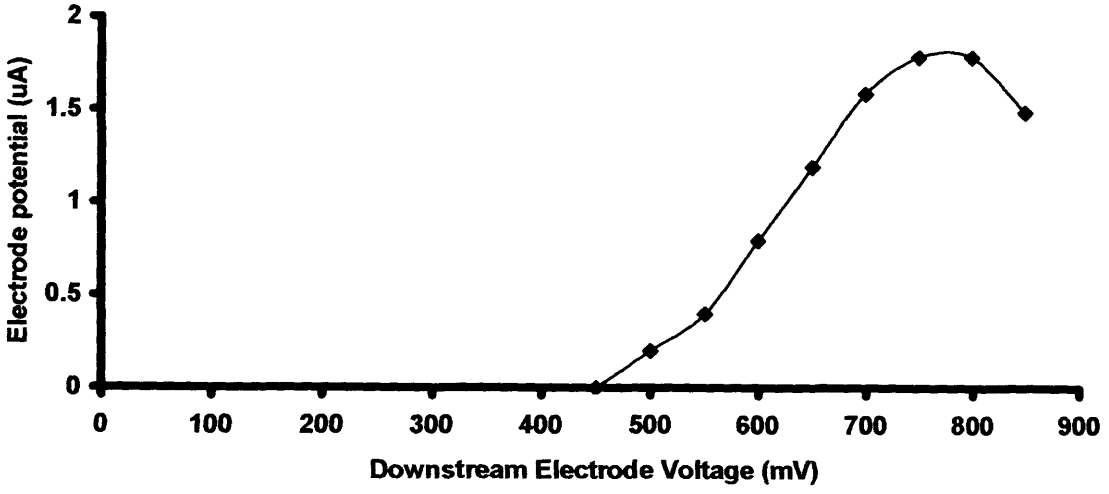


Figure 2.6. Voltamogram of GSH

GSH standards (10μM) were separated by reverse phase HPLC and detected by electrochemical detection. The upstream electrode was set at +100mV while the downstream electrode was set at potentials between +100 and +600mV. Optimal detection occurred when the downstream electrode was set at potentials between +800mV and +850mV.

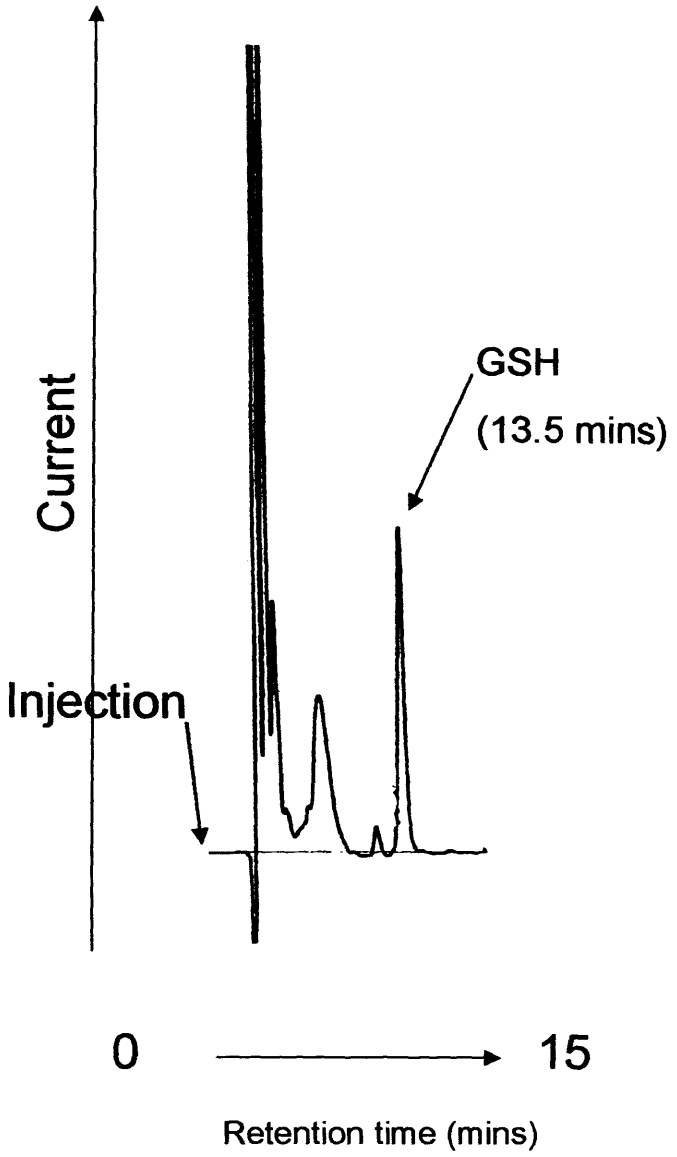


Figure 2.7. Chromatogram of an astrocyte sample

GSH from an astrocyte homogenate was extracted into 15mM OPA and quantitated by electrochemical detection following separation by reverse-phase HPLC.

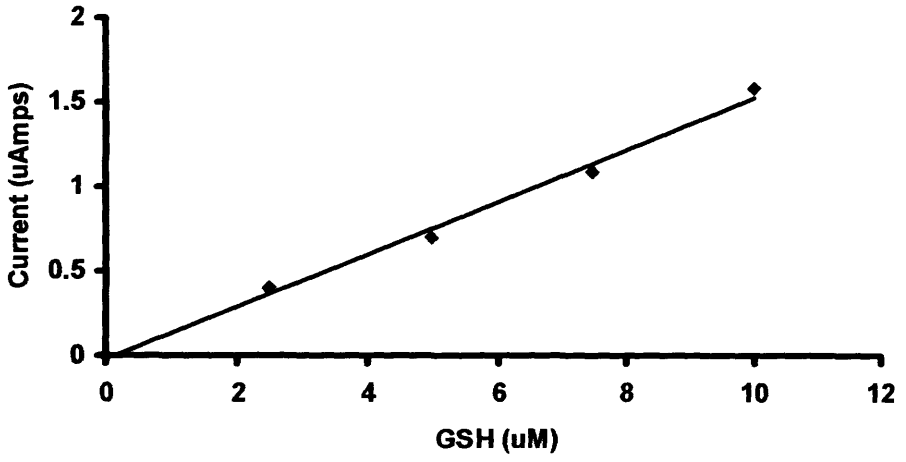


Figure 2.8. GSH standard curve

2.7. Protein determination

Sample protein concentration was determined by the Lowry method (Lowry *et al.*, 1951), using a Bio-Rad DC Protein Assay kit. To 200µl of sample (freeze thawed once; typically diluted 1/20 (v/v) in water) or BSA standards (0.2mg/ml in assay buffer), 100µl of alkaline copper tartrate and 800µl of Folin-Ciocalteu phenol reagent was added. Samples were vortexed and incubated at room temperature in the dark for 20 minutes. Absorbance was measured at 750nm on an Uvikon 941 spectrophotometer. Sample protein concentration was calculated from a BSA standard calibration curve (0-200µg/ml).

2.8. Statistical analysis

Linear regression of graphs was calculated using Microsoft excel.

Results are expressed as mean ± SEM for the number of independent cell culture preparations stated. Statistical significance of data was assessed using student's t-test or one-way ANOVA followed by the least significant difference test. $p < 0.05$ was considered significantly different compared to control. Where activities were expressed as a ratio, such as complex activity as a ratio to citrate synthase activity or percentages data were first transformed (Transformation = $\arcsin\sqrt{(\text{ratio})}$) to yield data with a normal distribution before statistical evaluation. (Gegg *et al.*, 2003).

Chapter 3

A spectrophotometric method for determining haem oxygenase activity in astrocytes

3.1 Introduction

Since haem oxygenase (HO) has been identified as an enzyme of possible importance in the antioxidant defences of the cell, its expression and activity have been studied in many different cell types. Antibodies to both HO-1 and HO-2 have been developed and protein expression in tissues and cells has been widely investigated. Recently an ELISA kit has been made available to detect HO in tissues and cell lysates. Activity of the enzyme is also important in assessing its possible antioxidant properties and in recent years a number of different assays have been developed to measure this. Most assay methods measure production of one of the end products of HO action, carbon monoxide, bilirubin, or iron (**Figure 3.1.**), and are measures of total HO activity. Radioactive methods have been used to follow the fate of labelled carbon into bilirubin (^{14}C haem to ^{14}C bilirubin)(Laitinen & Juvonen, 1995) and iron into free iron (^{55}Fe haem to ^{55}Fe) (Ferris *et al.*, 1999). Assays used to quantify CO production use gas chromatography (William Sunderman Jr. *et al.*, 1982, Vreman & Stevenson, 1988).

Many groups have used the formation of bilirubin to quantify HO activity using a spectrophotometric method. This was reported by Tenhunen *et al.*, in 1968 who first described the haem oxygenase enzyme system. This group had noted that in the intact organism haem and haemoglobin are converted nearly quantitatively to bilirubin. Suggestions were made as to how this conversion could come about, Lemberg (1956) suggested a coupled oxidation with ascorbate and molecular oxygen which *in vitro* leads to formation of bile pigment precursors. However

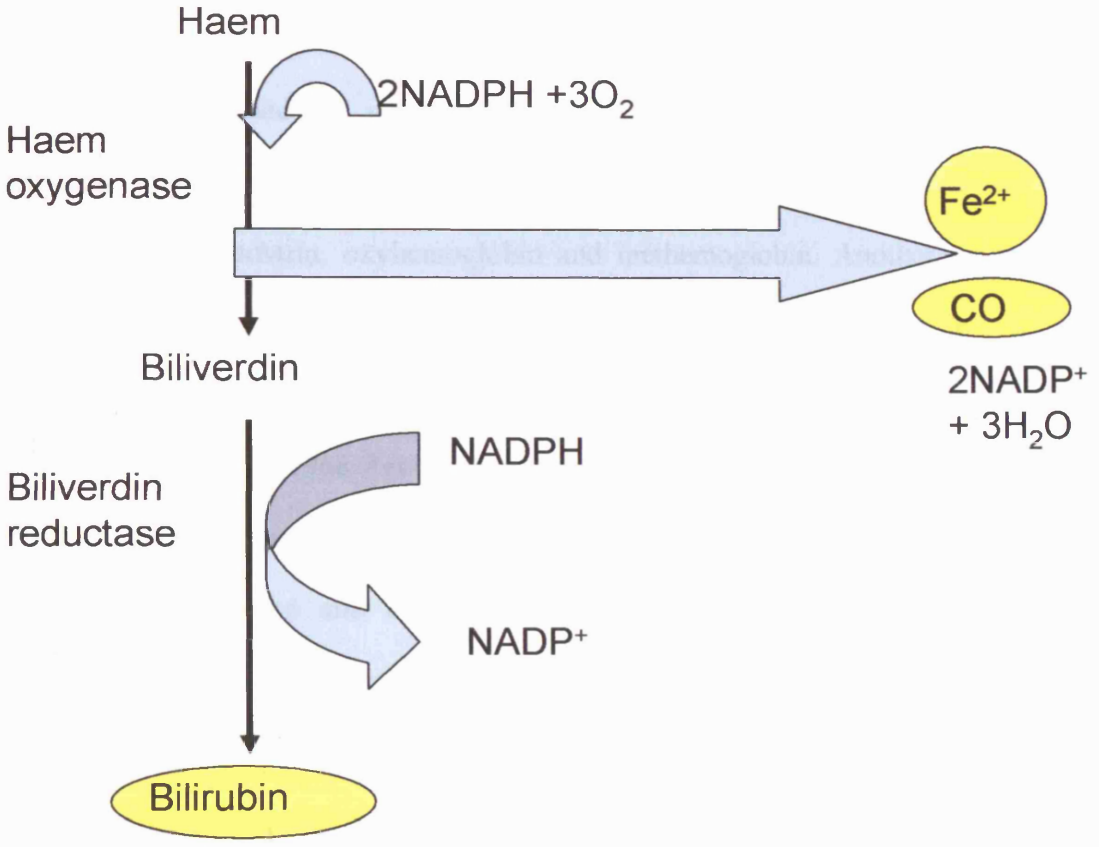


Figure 3.1. Schematic of the haem oxygenase/biliverdin reductase reaction.

Measurable products are shown on yellow backgrounds.

this was discounted as biliverdin produced by this method is a mixture of isomers whereas *in vivo* only the α-isomer of bliverdin is produced. Nakajima *et al.*, (1963) reported an enzyme called heme α-methenyl oxygenase which was discounted as it had an unusual specificity and acted only on pyridine hemochromogen, haemoglobin-haptoglobin complex, and myoglobin but was non-active with hematin, oxyhemoglobin and methemoglobin. Another reason that this enzyme did not seem a likely candidate for that conversion was the fact that bone marrow and spleen which are active in haem degradation showed almost no activity. In 1968 Tenhunen *et al.*, showed the production of bilirubin from haem via an enzymatic mechanism. This was done by incubating non-activated liver, spleen and kidney microsomes with methaemalbumin. The reaction required the presence of molecular oxygen and NADPH and the reaction product showed absorbance in the region of 460-470nm. This product was subsequently confirmed as bilirubin. Although microsomes alone were active in converting haem to bilirubin, activity was greatly enhanced by the addition of a 105,000 x g supernatant fraction. It was discovered that this fraction contained biliverdin reductase, which reduces biliverdin, the initial product of haem breakdown, to bilirubin. The method used by Tenhunen *et al.*, forms the basis of the spectrophotometric assay still used today. This assay is routinely used in many laboratories and was chosen for this investigation to quantify HO activity. The assay requires all the components for the conversion of haem to bilirubin; microsomes from the cells in question, where HO is located, substrate for the enzyme, an NADPH generating system, a source of biliverdin reductase and suitable buffer.

Aims

1. To validate this assay method for the quantitation of HO activity in primary rat astrocytes

2. To induce HO activity in primary rat astrocytes

3. To measure this activity using the spectrophotometric quantification of bilirubin.

3.2 Methods

3.2.1. Cell culture

Primary astrocytes were cultured as described in section **2.2.** On day 13 the media was removed from flasks and replaced with 10ml media containing 100μM hemin to induce HO activity. The flasks were incubated for a further 24 hours before the cells were harvested for assay.

3.2.2. Preparation of liver cytosol as a source of biliverdin reductase

Adult male Wistar rats (200-250g) were sacrificed by cervical dislocation and the liver immediately perfused with ice-cold 1.15% KCl solution to remove as much

of the blood from the liver as possible. The liver was then removed and finely chopped with scissors in 1 volume of homogenizing buffer (1.15% KCl -20mM Tris buffer pH 7.4). The liver was then homogenized in a teflon/glass homogeniser and centrifuged at 5000 x g for 20 minutes. At this time the lipid layer which had formed on top of the supernatant was removed and discarded and the supernatant transferred to a fresh centrifuge tube. The supernatant was then centrifuged at 105,000 x g for 1 hour. Once again the lipid layer was removed and the 105,000 x g supernatant removed to Eppendorf tubes. An aliquot of cytosol was removed for protein determination and the remainder frozen in liquid nitrogen and stored at -70°C until assay. In initial experiments the liver was removed and homogenised immediately without perfusion, however it is thought that this contributed to the lack of success in measuring HO activity at this time as haemoglobin in the cytosol can interfere with the assay.

3.2.3. Haem Oxygenase assay

Astrocytes were harvested (as described in section **2.2.3.3**.) and resuspended in 500µl HO buffer (100mM K_2HPO_4, 100mM KH_2PO_4, 2mM $MgCl_2$ pH 7.4). Samples were freeze/thawed in liquid nitrogen three times and sonicated on ice for 10 seconds to disrupt cell membranes. A 400µl aliquot of the sample was mixed with the following assay components; HO buffer, rat liver cytosol (5-7mgs), hemin (20µM final concentration, HO k_m for hemin 5µM, (Tenhunen *et al.*, 1969)), glucose-6-phosphate (G-6-P) (2mM final concentration) and glucose-6-phosphate dehydrogenase (0.75U/ml final concentration, k_m for G-6-P 36µM). The reaction tubes were vortexed and 0.8mM NADPH was added to start the

reaction. Tubes were incubated at 37°C in the dark for the appropriate time, and the reaction was stopped by adding 1ml ice cold chloroform and vortexing for 30 seconds. Samples were centrifuged at 500 x g for 10 minutes, after centrifuging 3 clear layers were evident in the sample tubes, a top aqueous layer, a middle layer which was comprised of solid lipids and cell material and a bottom organic layer into which any bilirubin had been extracted. The organic layer was removed to a quartz cuvette and the absorbance read between 464-530nm. The difference between the two wavelengths was calculated and HO activity was expressed in picomoles of bilirubin/mg/hr. (ε40mM-1.cm-1). The spectrophotometer was blanked using chloroform before each measurement.

In chloroform bilirubin has a maximal absorbance of 464nm, the HO assay uses 464-530nm to calculate bilirubin present as 530nm is the absorption at which bilirubin does not have any reading so is used as the baseline. In order to check this a 10μM solution of bilirubin was made up in bilirubin and the absorbance spectra was read. At the same time 10μM chloroform solutions of hemin and biliverdin were made up to measure their absorbance in case they could prove to interfere with absorbance results.

The above method was the one used during this investigation, however a number of difficulties were experienced initially. Methods used by Motterlini *et al.*, (1996), Maines (1996) and Iyer *et al.*, (1998) were investigated without success and it became apparent on investigating the assay components that some points were critical to the success of the assay. Firstly, as mentioned in section 3.2.2. the liver must be perfused before homogenisation so as to minimise haemoglobin interference in the assay, chloroform must be of spectrophotometric grade (intial

experiments were carried out using HPLC grade chloroform) and once the assay has been stopped with ice-cold chloroform, mixing must be extensive to extract bilirubin into the chloroform for measurement.

3.3. Results

3.3.1. Validation of HO assay method

Bilirubin was soluble in chloroform and absorbed maximally at around 464nm, there was no positive absorbance beyond 530nm (**Figure 3.2.**). Neither hemin nor biliverdin were soluble in chloroform (20μM and 10μM respectively).

Confluent astrocytes in $80cm^3$ flasks were treated with 100μM hemin for 24 hours. Cells were harvested as described in section (**2.2.2.3.**) and pooled into HO assay buffer. The protein concentrations were determined and samples were diluted in buffer to concentrations of 0.15, 0.25, 0.3, 0.5 and 1mg/ml. Samples were then assayed for HO activity (for 1 hour as described in Motterlini *et al.*, (1996)) as described in section 3.2. Using this method activity was found to increase linearly with protein concentration, up to 1mg protein per ml (R^2 = 0.9956, n=3).

In order to further validate the assay for use in rat astrocytes as isolated by the method described in section (**2.2.3.**) a time course experiment was carried out. Confluent astrocytes in $80cm^3$ flasks were treated with 100μM hemin for 24

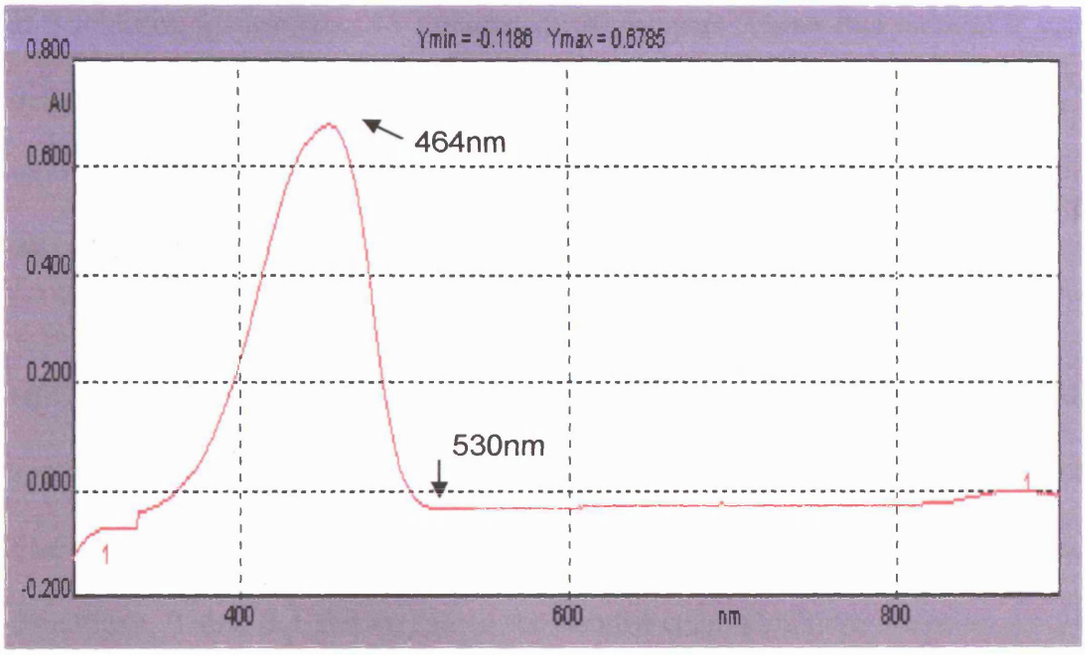


Figure 3.2. Bilirubin spectra

10μM bilirubin was dissolved in chloroform and the spectra recorded. Maximal absorbance is shown at around 464nm, no positive absorbance is seen after 530nm.

hours. Cells were harvested as described in section (**2.2.2.3.**). Protein concentrations were determined and 0.5mg protein was resuspended in assay buffer for each measurement. Samples were then assayed for HO activity as described in section (**3.2.**) but in this case the reaction was stopped, immediately, at 5 minutes, 10 minutes, 15 minutes, or 30 minutes. Using this method it was determined that bilirubin production increased linearly for periods of up to 15 minutes incubation ($R^2 = 0.9905$, n = 3-4). (**Figure 3.3**). Beyond this point there was no further increase in product formation.

HO was induced using hemin in order to measure activity, as the HO assay also measures bilirubin produced by any other isoforms of HO it could be expected that levels of HO activity due to the constitutive form may be present under basal conditions. **Table 3.1** shows values for control cells which were treated for 24 hours before harvest with fresh media only and 100μM hemin treated cells as shown previously in the validation assay.

3.4. Discussion

This chapter has shown a method for measuring HO activity that has been validated in primary rat astrocytes. At the time this investigation was started, although HO-1 protein had been shown to be expressed in astrocytes, activity had not been determined. This is an assay that requires no specialist equipment or costly substrates and so has some advantages over other methods. It is important however to optimise experimental conditions for the cells/tissue being analysed, particularly with regards to time.

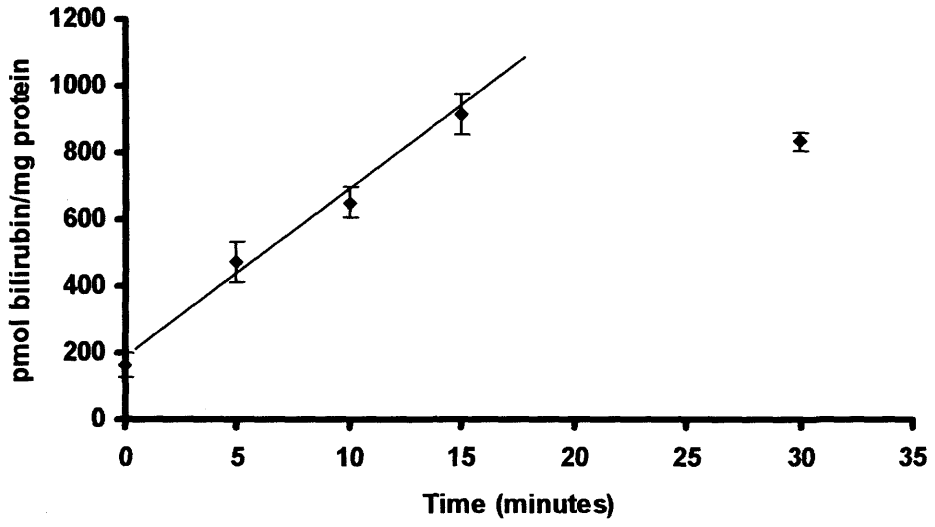


Figure 3.3. HO activity against time

Astrocytes treated with 100μM hemin were assayed using incubation times of up to 30 minutes to determine linearity with time. 0.5mg protein assayed, n = 3-4.

Haem oxygenase activity in rat astrocytes pmol bilirubin/mg/min	
Control	21.3 ± 5.8
Hemin treated (100μM)	61.1 ± 5.3*

Table 3.1. Haem oxygenase activity in rat astrocytes

Rat astrocytes were treated for 24 hours with media alone or 100μM hemin and HO activity measured. Hemin treated astrocytes show a significantly greater amount of bilirubin production. Data was mean ± SEM (n = 4 independent cell preparations). Statistical significance was determined by students t-test. * $p < 0.05$.

Whilst investigating the spectrophotometric assay for bilirubin production it became apparent that there were a number of factors for consideration as previously mentioned, the liver cytosol preparation and grade of chloroform in particular. A recent paper (McNally *et al.*, 2004) has highlighted other considerations in optimising this assay. This group report that, although it is possible to measure bilirubin in the aqueous phase, the solubility of bilirubin in chloroform is much greater. Bilirubin has a low solubility in phosphate buffer at pH 7.4 with a tendency to exist as a colloidal sol or to form large flocculants. The true solubility of bilirubin in phosphate buffer has been reported to be less than 0.1μM (Lee & Gartner., 1976). However in chloroform the solubility is reported to be up to 20mM, although at this concentration it exists as a particulate solution. Using a chloroform extraction also reduces the effect that hemin can have on the assay. McNally *et al*, (2004) report that although hemin is required in the assay as a substrate, too high a concentration can reduce the sensitivity of the bilirubin measurement, but too low a concentration affects the efficiency of the enzymatic reaction. A stepwise reduction in apparent bilirubin content with increasing hemin concentration above 25μM in 0.1M phosphate buffer is seen, however in chloroform this is not seen until hemin concentrations are above 50μM. It has also been reported (Tenhunen, 1972) that large amounts of protein in the reaction inhibit the function of both haem oxygenase and biliverdin reductase. Indeed increasing the amount of biliverdin reductase protein in the assay above 6mg did not increase the amount of bilirubin formed (up to 15mg) in the reaction and so was used as an optimum amount.

3.5. Conclusions

The HO method described above has been validated for use in rat astrocytes and will be used to determine levels of HO activity under various conditions to determine the role of HO activity in NO mediated mitochondrial damage. This assay measures all HO activity (HO-1, HO-2 and HO-3) however since HO-2 is only thought to be responsive to adrenal glucocorticoids (Maines, 1997) and HO-3 has only marginal levels of catalytic activity compared to HO-2 (McCoubrey at al., 1997) increases in HO activity brought about by treatments to the astrocytes (other than glucocorticoids) are most likely to be attributable to HO-1.

Chapter 4

The effects of hemin on HO induction, GSH status and mitochondrial function in astrocytes

4.1. Introduction

In the mature mammalian brain astrocytes constitute nearly half of the total cells (Song *et al.*, 2002). In recent years it has become clear that far from being only the "glue" that supports the brain structure, astrocytes perform a number of crucial functions. Apart from providing structural support for neurons, astrocytes are now known to be able to modulate the environment around neurons, release neuronal growth factors and help maintain the barrier between blood and brain. New roles for astrocytes are still being discovered, for example, in 2002 Song *et al.*, reported that certain astrocytes control neurogenesis. Defense against oxidative stress is another important role for astrocytes and it has been shown that they are able to protect neurones against oxidative stress via upregulation of glutathione synthesis. Glutathione is present at high concentration in the brain and acts as a major antioxidant (Meister, 1988, Makar *et al.*, 1994 and Nakamura *et al.*, 1997). It has been suggested that oxidative stress can influence levels of GSH by upregulating enzyme activity of glutamate-cysteine ligase (GCL) (Iwata-Ichikawa *et al.*, 1999; Gegg *et al.*, 2005).

Haem oxygenase (HO) specific activity in the brain exceeds that of various organs including the liver (Trakshel *et al.*, 1986; Maines, 1992); and under normal conditions this activity is attributable to HO-2 and is mainly expressed in neuronal populations (Ewing & Maines, 1992) and the endothelial lining of the blood vessels (Ewing *et al* ., 1994). However, in response to stress HO-1 is expressed at high levels in glial cell populations and macrophages (Ewing & Maines, 1991; Ewing *et al.*, 1992; Ewing & Maines, 1993; Geddes *et al.*, 1996;

Turner *et al.*, 1998), making it possible that HO-1 induction in glial cells which include astrocytes is an important response to stress.

Central nervous system haemorrhage exposes glial cells to high micromolar concentrations of haem (Letarte *et al.*, 1993) in the form of haemoglobin, which is able to spontaneously, nonenzymatically convert to methaemoglobin (Huang *et al.*, 2002), from which hemin readily dissociates (Bunn & Jandl., 1968; Hebbel & Eaton., 1989).

Hemin also known as ferriprotoporphyrin IX (**Figure 4.1**.), differs from haem in that the central iron atom in this molecule is in the ferric (Fe^{3+}) state. The porphyrin structure is however, the same as haem and it is therefore a substrate for the haem oxygenases. Due to the oxidative nature of the hemin molecule it is also a very effective inducer of HO-1. At high micromolar concentrations of hemin such as may be found in intracranial haematomas, hemin has been found to be toxic to various cell types including porcine aortic endothelial cells (Balla *et al.*, 1992), myocytes (Bhoite-Soloman *et al.*, 1993), epithiloid sarcoma cells (Braverman *et al.*, 1995) and an African green monkey kidney epithelial cell line (da Silva *et al.*, 1996). However the mechanisms underlying the toxicity are unclear. Hemin accumulates in membranes, and toxic effects may be due to interactions with membrane lipids and /or proteins (Shaklai *et al.*, 1985; Leclerc *et al.*, 1988). It is also possible that due to hemin's induction of HO-1 and subsequent action of the enzyme, iron could be released from the hemin molecule and take part in potentially deleterious oxidative reactions. Mechanisms to prevent a toxic overload of iron on the cell are available such as ferritin induction which is said to be upregulated in concert with HO-1 induction

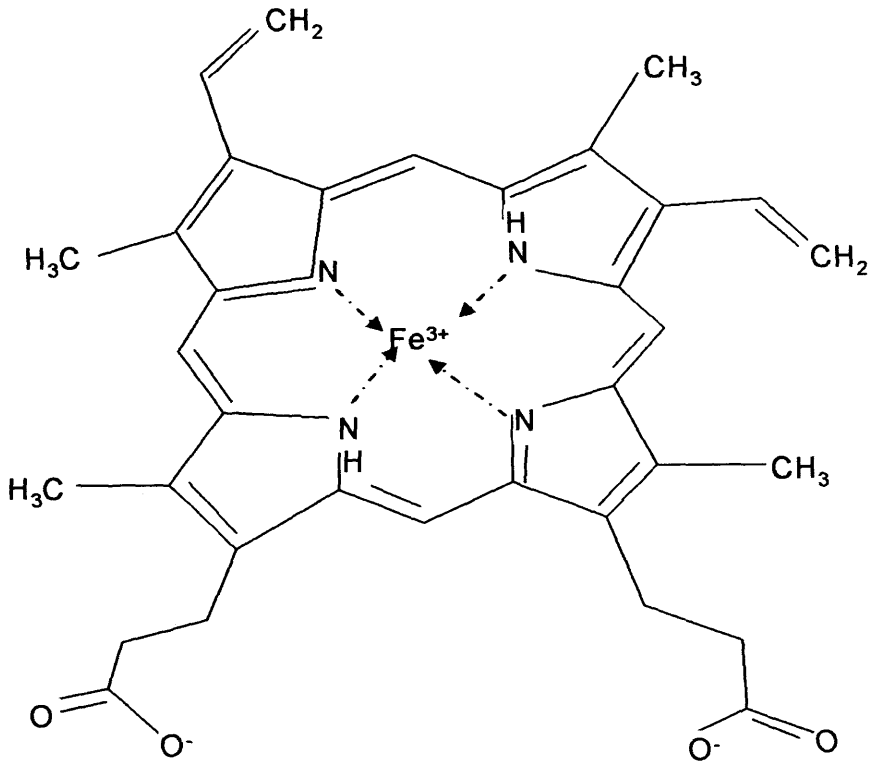


Figure 4.1 Structure of hemin

Adapted from http://www.mpcfaculty.net/

(Eisenstein *et al.*, 1991) however it is possible that in situations where haem overload occurs such as haemorrhage, the ability of ferritin to sequester iron may be overwhelmed. Iron release from hemin can also occur via non HO mediated methods, since hemin can rapidly react with peroxides resulting in its degradation (Kremer, 1989). Increased superoxide production and its conversion to H_2O_2 by superoxide dismutase may increase iron release from hemin via this mechanism.

Although there are studies that have shown hemin toxicity at low concentrations, not all reports agree with this. Clark *et al.*,(2000) show bilirubin mediated protection against glucose oxidase (GOX) treatment, an oxidant challenge at hemin concentrations up to 200μM in bovine vascular smooth muscle cells (Clark *et al.*, 2000). Highest HO activity was seen using 100μM hemin even though highest HO-1 protein expression was seen using 200μM hemin. The higher HO activity correlated with an improved protective effect after GOX treatment. Other investigations have also shown protective effects of hemin mediated HO induction both *in vivo* and *in vitro* (Chen *et al.*, 2004; Hangaishi *et al.*, 2000; Le *et al.*, 1999; Aizawa *et al.*, 1999). Hemin has proved to be a useful tool to study HO induction, however it must be remembered that enzyme induction is not the only effect of this molecule.

In order to study the effects of an enzyme it is also useful to be able to inhibit it. Inhibitors of HO have been viewed with increasing interest in recent years, not only as a tool to study the enzyme but also as therapeutic agents to prevent hyperbilirubinemia in newborns (Kappas, 2004). HO inhibitors are synthetic haem analogues with a metal ion other than iron inserted into the centre of the

porphyrin ring, and inhibit by competitive inhibition. The central metal atom does not bind molecular oxygen and this means that it cannot be degraded (Drummond & Kappas, 1981). Two of these analogue inhibitors are zinc protoporphyrin IX and tin protoporphyrin IX (**Figure 4.2**.), which have both been used to study the effects of HO inhibition both *in vivo* and *in vitro* (Zakhary *et al.*, 1996; Grunemar & Ny, 1997; Doi *et al.*, 1999; Cavicchi *et al.*, 2000).

In view of the physiological role that hemin could play in the brain under certain conditions and the fact that it is such a potent inducer of HO-1, this molecule was used as a model of HO-1 induction in primary rat astrocytes.

The aims of this chapter were to;

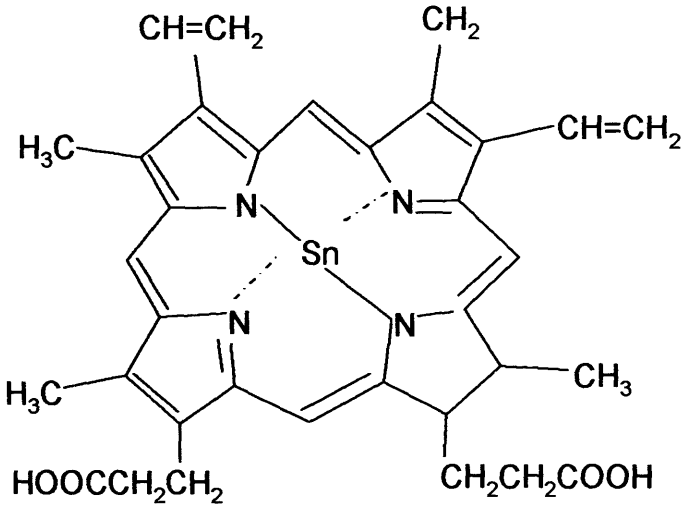
1. Examine the effects of hemin treatment on cultured rat astrocytes by measuring cell viability, HO-1 expression and HO activity

2. Since it was intended to go on to study the effects of HO induction on IFNγ/LPS activated astrocytes, the effect of hemin mediated induction of HO was also examined on the following parameters which may be affected by IFNγ/LPS activation; iNOS expression, nitrite/nitrate in cell culture media as an index of iNOS activity, cellular GSH levels and mitochondrial respiratory chain enzyme activities.

3. Treat cells with ZnPPIX and SnPPIX and assess viability to decide on the most suitable inhibitor to use.

4. Treat cells with the most suitable inhibitor and assess amount of HO inhibition and investigate what effects this inhibition has on the parameters listed in 1.

a)



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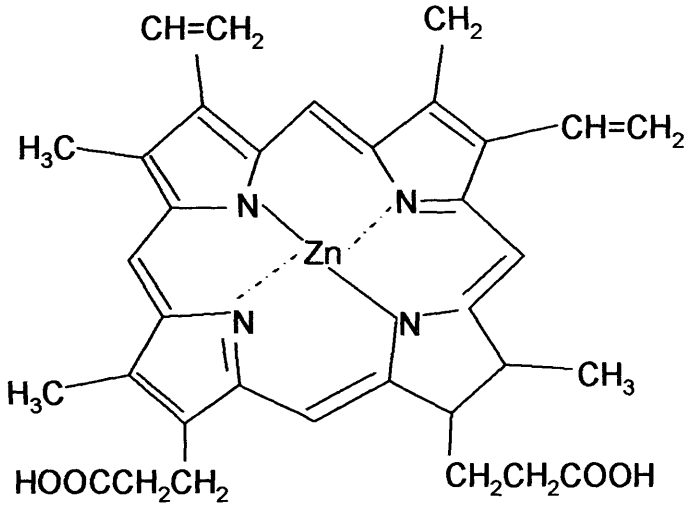


Figure 4.2. Structure of HO inhibitors

Structure of a) tin protoporphyrin IX (SnPPIX) and b) zinc protoporphyrin IX (ZnPPIX) (adapted from http://www.alexis-corp.com/porphyrins)

4.2. Methods

4.2.1. Cell culture

Primary astrocytes were cultured as described in section 2.2 and treated with 100μM hemin, for 3,6,18 and 24 hours. Control cells were treated with medium only for the same times. In other experiments cells were treated with 20μM ZnPPIX or SnPPIX for 24 hours.

4.2.2. MTT reduction

Chemical reduction, by cells, of the redox dye MTT was used as an indication of cellular redox capacity, which in turn may be interpreted as a measure of cell viability (Regan *et al.*, 2002; Goldstein *et al.*, 2003). The tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is taken up into cells and reduced by a cellular dehydrogenase enzyme to yield a purple formazan product which is largely impermeable to cell membranes, thus resulting in its accumulation in healthy cells. Solubilisation of the cells results in the liberation of this product which can then be detected using a colorimetric assay. 0.12μM MTT was made up in HBSS and 1ml of this solution was added to control astrocytes and astrocytes treated with 100μM hemin, 20μM ZnPPIX or 20μM SnPPIX for 24 hours.The cells were incubated at 37°C in the dark for 1 hour to allow reduction to occur. MTT was reduced to purple formazan crystals, which precipitated on the bottom of the well. The supernatant was removed and the crystals of formazan were dissolved in 1ml of dimethylsulphoxide (DMSO). The absorbance of the samples (using DMSO as a blank) was read at 540nm using a

Uvikon 941 spectrophotometer. The absorbance of treated samples was expressed as a percentage of absorbance in control samples.

4.2.3. HO assay

Bilirubin production by astrocytes in control, hemin, inhibitor or hemin + inhibitor treated cells was determined as described in section **3.2.3**.

4.2.4. Spectrophotometric enzyme assays

Complexes I, II+III and IV of the ETC and citrate synthase (CS) were assayed in cultured astrocyte homogenates as described in section **2.3**

Lactate dehydrogenase release from cultured astrocytes was measured as described in section **2.3.5**.

4.2.5. Total nitrite/nitrate

Accumulation of the stable breakdown products of NO breakdown nitrite and nitrate was measured as described in section **2.4.1**. Since the Griess reaction is a colourmetric reaction it was necessary to check that hemin or inhibitors used during the investigation did not affect results. This was done by making up

standard concentrations of $NaNO_2$ and $NaNO_3$ in media containing hemin or inhibitor at the concentration used in the treatment, and then performing the assay as usual. Conversion from nitrate to nitrite was also recorded to ensure that treatments were not interfering with this process.

4.2.6. GSH quantification

GSH content in cultured astyrocyte homogenates was analysed by reverse-phase HPLC as described in section 2.6.

4.2.7. Western blots for HO-1 and iNOS

Expression of H0-1 and iNOS proteins was detemined by subjecting samples to SDS-PAGE to separate the proteins then using antibodies to either iNOS and HO as described in section 2.5. To show equal loading of protein in each lane an actin antibody was also used.

4.2.8. Protein determination

Sample protein concentration was determined using the Lowry method as described in section **2.7**.

4.3. Experimental protocols

Hemin and inhibitor treatments

Hemin , ZnPPIX or SnPPIX were dissolved in a minimal amount of 0.1M NaOH and made up to a stock concentration of 20mM with astrocytic medium, pH was checked to ensure that the buffering ability of the media was sufficient to buffer this concentration of NaOH, (< 0.1 pH unit difference) . Final treatment concentration was 100μM for hemin, and 20μM for ZnPPIX and SnPPIX in astrocytic medium. Control astrocytes were treated with medium alone. Astrocytes were treated for 3,6,18, or 24 hours before being harvested for assay.

4.4 Results

4.4.1. Cell viability after hemin or inhibitor treatment

Before assessing the effect of hemin or inhibitors on the various parameters chosen it was necessary to establish that the treatment itself would not cause cell

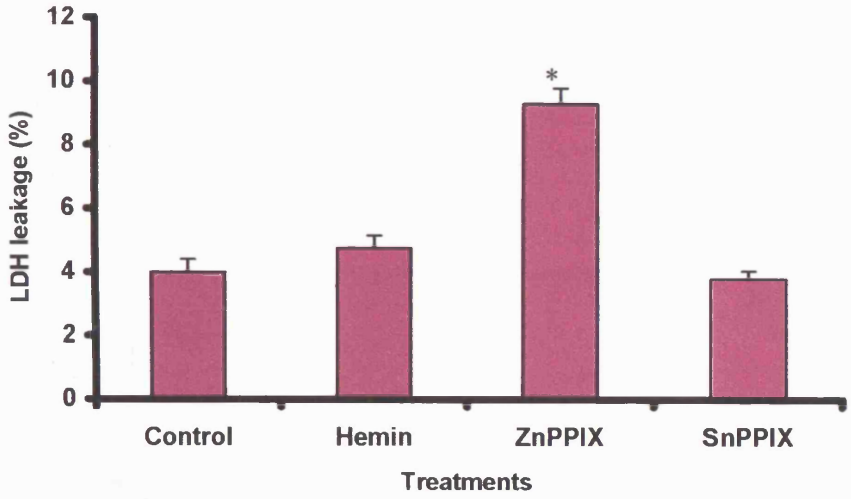
death. LDH leakage and MTT reduction were used as indicators of cell viability. Astrocytes were treated for 24 hours with 100μM hemin, 20μM ZnPPIX or 20μM SnPPIX in media, control cells were treated with media alone. 100μM hemin treatment or 20μM SnPPIX treatment for 24 hours did not cause significant LDH leakage compared to control **(Figure 4.3a)** (control, 4% ± 0.4%; hemin , 4.75% ± 0.75%: SnPPIX, 3.8% ± 0.25%(n = 4)). Hemin or SnPPIX treatment also did not significantly affect the astrocytes ability to reduce MTT **(Figure 4.3b)** (control, 100%; hemin, 96.4% ± 2.9%; SnPPIX , 99.5% ± 0.4% (n = 4)). ZnPPIX, however affected LDH leakage and MTT reduction in a significant manner when measured against controls **(Figure 4.3a)** (LDH leakage: control, 4% ± 0.4%; ZnPPIX, 9.3% ± 0.5% (n = 4) $p<0.05$) **(Figure 4.3b)** (MTT reduction; control 100%; ZnPPIX, 58.8% ± 10.5% (n = 4) $p<0.05$).

Since astrocyte viability was significantly affected by ZnPPIX at the concentration used SnPPIX was used in all further inhibiton experiments.

4.4.2. Expression of HO-1 and iNOS after hemin treatment

Treatment of astrocytes with 100μM hemin caused enhanced expression of HO-1 protein at all time points compared to control (Representative blot shown in **Figure 4.4**). Expression was increased by 3 hours of treatment and continued to increase up to 24 hours. Control levels of expression did not increase during the experiment. iNOS expression was not visible in either the control or treated cells although confirmation that the antibody was able to detect this was confirmed by the use of a positive control **(Figure 4.4)**. The positive control stimulated with

a)



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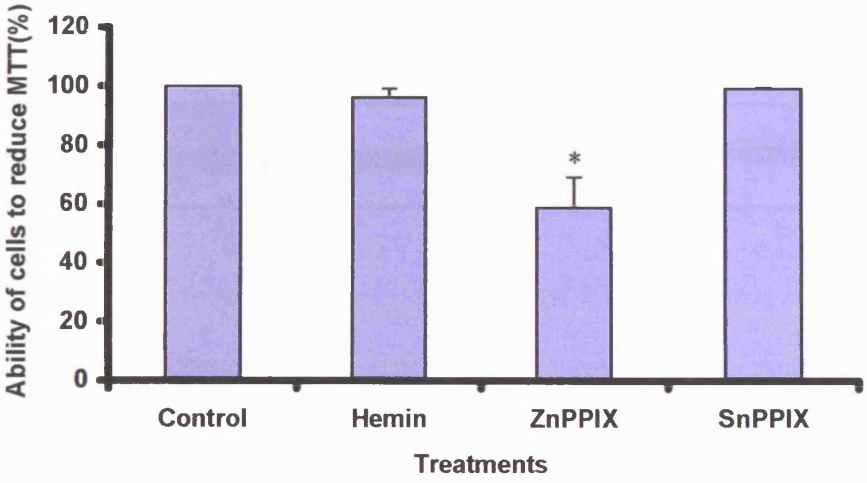


Figure 4.3. Measures of astrocyte viability after hemin and inhibitor treatment.

Astrocytes were treated with 100μM hemin, 20μM ZnPPIX, 20μM SnPPIX, or media alone (control) for 24 hours. LDH leakage and the ability of cells to reduce MTT were then analysed. Hemin or SnPPIX treatment did not significantly affect astrocyte viability compared to control as assessed by these parameters. ZnPPIX caused a significant decrease in cell ability to reduce MTT and a significant increase in LDH leakage against controls. Data are mean ± SEM (n = 4). Data was statistically evaluated by one-way ANOVA followed by least significant difference test. * $p < 0.05$.

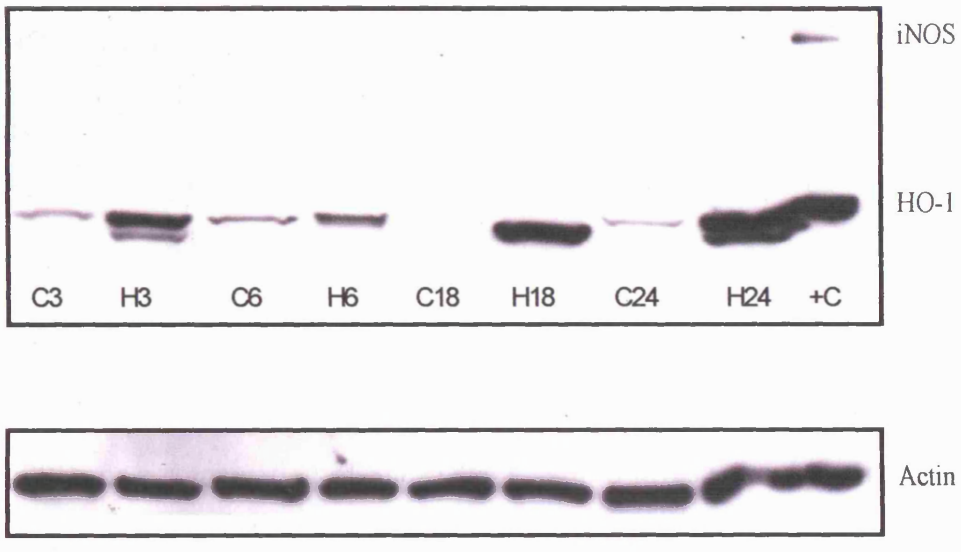


Figure 4.4. Western blot of HO-1 and iNOS from astrocytes exposed to hemin

Astrocytes were treated with media alone (C), or 100μM hemin (H) for 3,6,18 or 24 hours. Protein from astrocytes was electrophoresed via SDS-PAGE, transferred to a nitrocellulose membrane and probed with antibodies to HO-1 and iNOS. Following visualisation the membrane was probed with an anti actin antibody to show equal loading of proteins. +C is a positive control for iNOS, a murine macrophage cell line, RAW264.7 lysate (LPS and γ-interferon stimulated).

IFNγ + LPS also showed strong expression of HO-1 protein. Treatment of astrocytes with the HO inhibitor SnPPIX also showed a strong expression of HO-1 protein, **Figure 4.5** shows this expression to be similar to that found when cells are treated with hemin + SnPPIX.

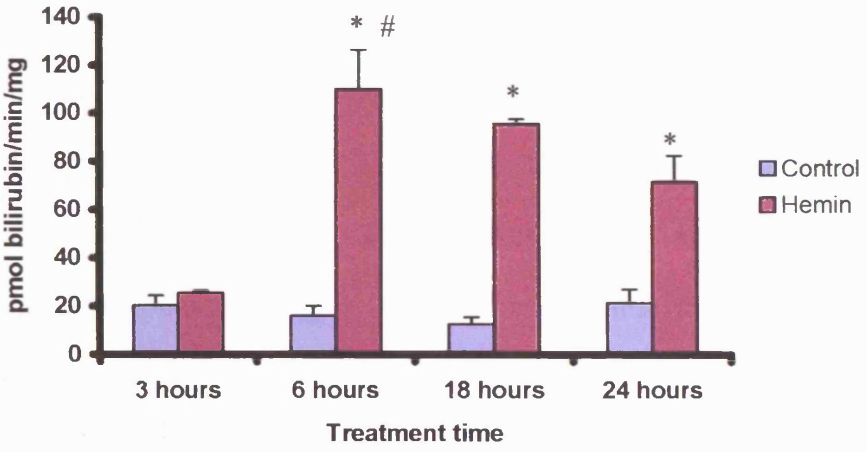
4.4.3. HO activity after hemin and HO inhibitor treatment

Bilirubin production as an index of HO activity was measured in astrocytes grown to confluency in $80cm^3$ flasks and treated on day 13 with media alone (control) or media supplemented with 100μM hemin. Treatments were stopped after 3,6,18 and 24 hours. Some flasks were treated with the HO inhibitor SnPPIX or a combination of SnPPIX + hemin to investigate the efficacy of the inhibitor. Activity in control cells did not change significantly at any time point **(Figure 4.6a)**. After 6 hours of hemin treatment enzyme activity did become significantly higher than that of controls or 3 hour hemin treated cells (hemin treated 6 hours, 110 ± 16.7 pmol/min/mg protein; hemin treated 3 hours, 25.4 ± 1.1 pmol/min/mg protein, $p < 0.05$, n = 3-5). After 18 hours of hemin treatment enzyme activity, although still significantly higher than controls was slightly decreased and this trend was continued at the 24 hour timepoint. At the 24 hour timepoint enzyme activity was significantly lower than at 6 hours (hemin treated 24 hours,71.6 ± 11.1 pmol/min/mg protein; hemin treated 6 hours, 110 ± 16.7 pmol/min/mg protein, p 0.05, n = 3-5) **(Figure 4.6a),** but still remained significantly higher than controls.

Figure 4.5. Western blot of HO-1 protein from cells exposed to an inducer and an inhibitor of HO-1

Astrocytes were treated with media alone (control), media supplemented with 20μM SnPPIX or media supplemented with SnPPIX + 100μM hemin. Cells were lysed and subjected to SDS-PAGE , transferred to a nitrocellulose membrane and probed with antibody to HO-1. The membrane was also probed with an actin antibody to show equal loading of protein.

a)



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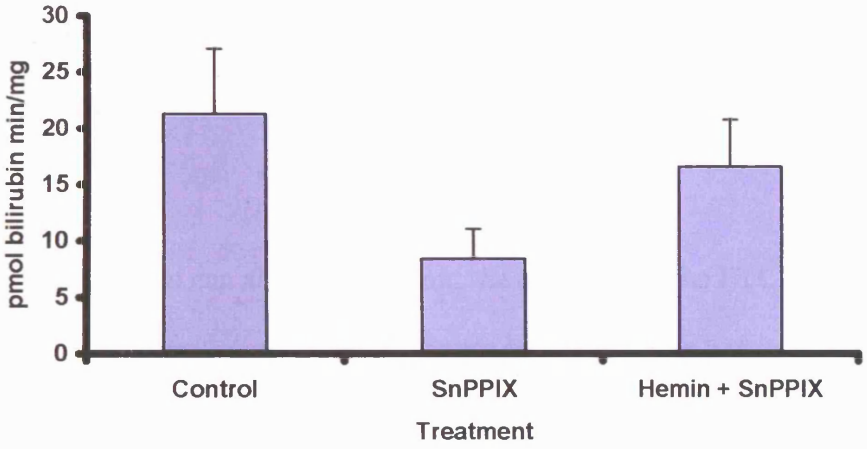


Figure 4.6. Haem oxygenase activity with time and inhibition after hemin treatment

Astrocytes were treated with medium alone (control) or medium containing 100μM hemin for 3 to 24 hours (a). Cells were also treated with 20μM SnPPIX alone or100μm hemin + SnPPIX for 24 hours (b). Haem oxygenase activity in these cells was then measured. HO activity was significantly higher in cells treated for 6, 18 and 24 hours compared to controls (*), HO activity in cells treated with hemin for 6 hours was also significantly higher than activity in cells treated for 24 hours (#). Values are mean ± SEM (n = 3-5 independent cell preparations). Cells treated with SnPPIX or SnPPIX + hemin for 24 hours showed HO activity levels which were not significantly different to controls (n = 3 – 4). Data was statistically evaluated by one-way ANOVA followed by least significant difference test. $p < 0.05$.

Treatment of astrocytes with SnPPIX for 24 hours did not cause bilirubin production to differ significantly from control levels (**Figure 4.6b**) (control 24hrs, 21.3 ± 5.8 pmol/min/mg protein; SnPPIX 24hrs, 10.2 ± 3.4 pmol bilirubin/min/mg protein, (n = 3)) likewise, the treatment of cells with hemin to induce HO-1 activity and an HO inhibitor, SnPPIX showed bilirubin levels comparable to control (**Figure 4.6b**) (control 24hrs 21.3 ± 5.8 pmol/min/mg protein ; SnPPIX + hemin 24hrs 16.6 ± 4.2 pmol/min/mg protein, (n = 3)).

4.4.4. Mitochondrial electron transport chain enzyme activity after hemin or HO inhibitor treatment

Given that hemin can act as an oxidant, the activities of the ETC complexes were assayed in astrocytes after hemin treatment to determine any effects. Activities were also determined after cells had been treated with an HO inhibitor alone or together with hemin. Astrocytes seeded onto 6-well plates were treated with media alone (control) or media supplemented with 100μM hemin for 3,6,18 or 24 hours. Cells were also treated with 20μM SnPPIX alone or SnPPIX + 100μM hemin. Hemin treatment or SnPPIX treatment alone did not result in any significant changes in respiratory chain enzyme activity at any time point using either protein or citrate synthase as a baseline (**Table 4.1).** Treating astrocytes with SnPPIX + hemin, however caused a significant increase in complex I activity against 24 hour control values whether assessed against a protein baseline or against citrate synthase (**Table 4.2 and Figure 4.7**) (vs protein:

Hours	Treatment	Comp I nmol/min/mg	vs citrate synthase	Comp II/III nmol/min/mg	vs citrate synthase	Comp IV *k*/min/mg	vs citrate synthase *k*/nmol	Cit Synth nmol/min/mg
3	Control	31.7 ± 3.1	0.203 ± 0.020	14.3 ± 0.4	0.091 ± 0.003	2.05 ± 0.06	0.013 ± 0.003	156.0 ± 2.1
	Hemin	31.1 ± 2.5	0.222 ± 0.008	13.0 ± 1.4	0.092 ± 0.007	2.09 ± 0.03	0.015 ± 0.001	141.6 ± 4.8
6	Control	31.9 ± 1.1	0.221 ± 0.018	16.1 ± 1.7	0.109 ± 0.008	2.27 ± 0.25	0.016 ± 0.002	148.2 ± 15.7
	Hemin	36.4 ± 2.1	0.227 ± 0.005	16.1 ± 2.3	0.090 ± 0.011	2.40 ± 0.24	0.018 ± 0.001	138.6 ± 24.4
18	Control	34.1 ± 0.2	0.256 ± 0.028	17.1 ± 5.3	0.107 ± 0.033	2.26 ± 0.13	0.015 ± 0.001	160.0 ± 2.0
	Hemin	31.4 ± 5.2	0.222 ± 0.037	16.2 ± 5.8	0.116 ± 0.021	2.25 ± 0.13	0.016 ± 0.001	143.7 ± 13.4
24	Control	32.2 ± 1.8	0.201 ± 0.009	15.0 ± 2.3	0.094 ± 0.013	2.12 ± 0.22	0.019 ± 0.003	160.5 ± 9.5
	Hemin	29.0 ± 1.2	0.184 ± 0.011	19.3 ± 0.8	0.131 ± 0.021	2.44 ± 0.28	0.015 ± 0.001	158.1 ± 3.6

Table 4.1 The effect of hemin on the ETC in astrocytes

Astrocytes were treated with medium alone (control) or medium supplemented with 100μM hemin for 3-24 hours. Following treatment cell homogenates were assayed for ETC complex activity. Data were expressed against protein and citrate synthase No significant differences were seen in any of the complexes compared to time matched controls after hemin treatment. Data are mean ± SEM (n = 3).

Treatment	Comp I nmol/min/mg	Comp II/III nmol/min/mg	Comp IV k/min/mg	Citrate Syn nmol/min/mg
Control	32.2±1.8	15.0±2.3	2.12±0.22	160.5±9.5
SnPPIX	32.6±4.4	11.4±0.8	1.63±0.18	127.0±17.4
Hemin+SnPPIX	75.2±5.1*	15.5±3.2	1.96±0.77	146.9±6.3

Table 4.2.

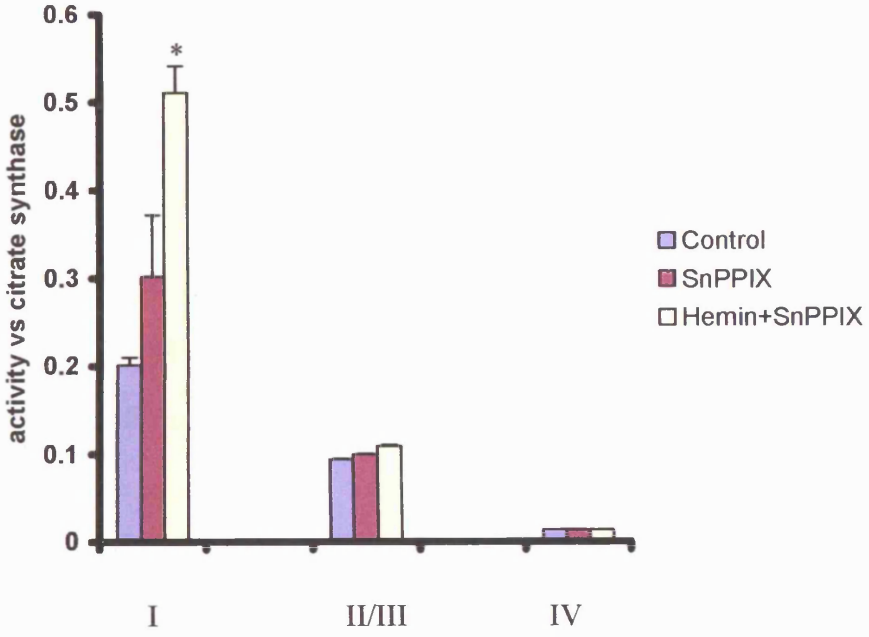


Figure 4.7. The effect of inhibiting HO on the ETC

Astrocytes were treated with medium alone (control), 20μM SnPPIX or 100μM hemin plus SnPPIX. Cells were homogenised and ETC enzyme activities measured. Activities were expressed against protein, or specific activities expressed against citrate synthase which have no units except for complex IV (expressed as *k*/nmol). Hemin + SnPPIX treatment caused a significant increase in complex I activity. Values are mean ± SEM (n = 3-5 independent cell preparations). Statistical significance was determined by one-way ANOVA followed by least significant difference test. * $p < 0.05$.

control 24hrs, 32.2 ± 1.8 nmol/min/mg protein; SnPPIX+hemin, 75.2 ± 5.1 nmol/min/mg protein, (n = 3) $p < 0.01$).

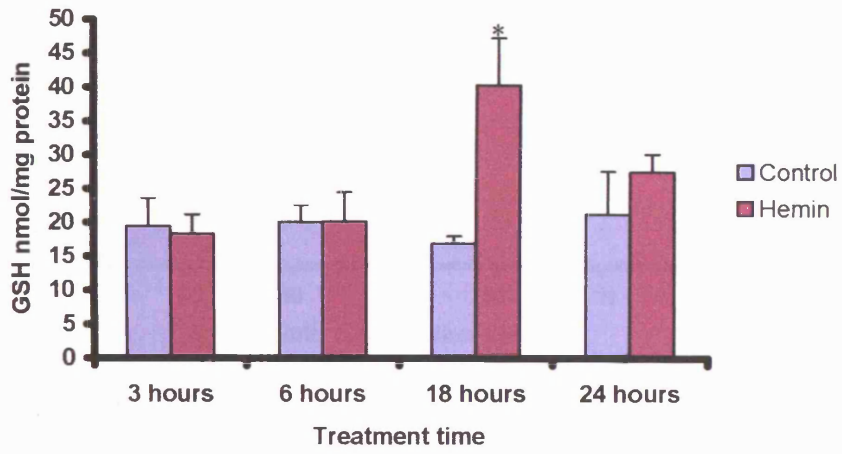
4.4.5. GSH levels after hemin treatment

Hemin treatment did not significantly affect GSH levels as compared to control at the 3 and 6 hour treatment time. After 18 hours there was a significant increase in GSH levels in treated cells **(Figure 4.8a)** (control 16.9 ± 1.1 nmol/mg protein ; hemin treated 40.5 ± 6.9 nmol/mg/protein (n = 5-6 independent cell preparations) $p < 0.01$). At 24 hours GSH were still elevated but to a lesser extent. Treating cells with SnPPIX alone did not have any significant effects on GSH levels compared to control **(Figure 4.8b)**, likewise SnPPIX + hemin did not have a significant effect on GSH levels compared to 24 hour controls **(Figure 4.8b).**

4.4.6. Nitrite/nitrate levels after hemin treatment

Hemin and SnPPIX were used to prepare a standard curve for the Griess assay as described in section **4.2.5**. The standard curve showing conversion of nitrate to nitrite is shown in **Figure 4.9**, and shows conversion of nitrate to nitrite when media was supplemented with 100μM hemin **(Figure 4.9a)** and when media was supplemented with 20μM SnPPIX .Conversion of nitrate to nitrite was not affected by the presence of 100μM hemin or 20μM SnPPIX. The comparison of

a)



b)

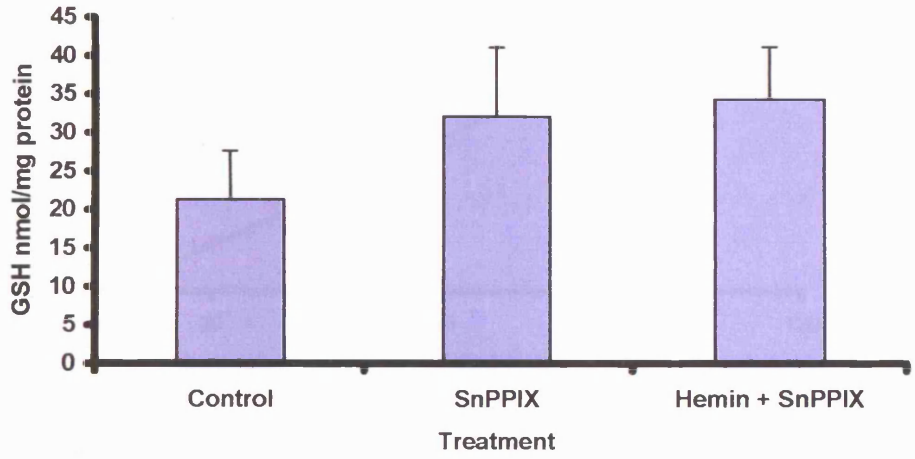
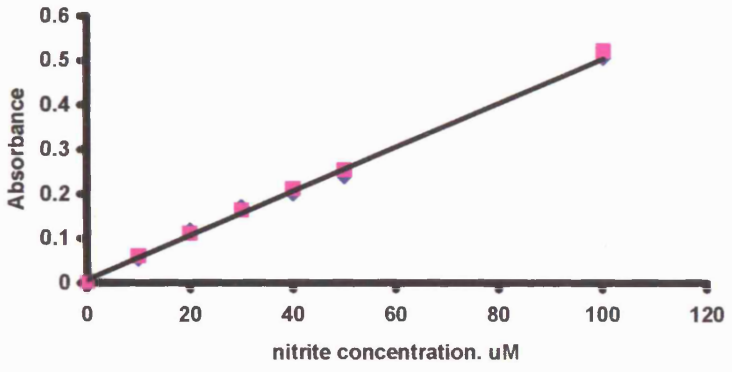


Figure 4.8. GSH levels in astrocytes treated with hemin over time and an HO inhibitor

Astrocytes were treated with media alone (control) or media containing 100μM hemin for 3 to 24 hours. Cells were also treated with 20μM SnPPIX alone or 20μM SnPPIX + 100μM hemin for 24 hours. GSH levels in cell homogenates were then measured. GSH levels in cells treated with 100μM hemin for 18 hours were significantly higher than levels at any other control point, or levels after 3 and 6 hours of hemin treatment. Values are mean ± SEM (n = 4 -6 independent cell preparations). Data was statistically evaluated by one-way ANOVA followed by least significant difference test. * $p < 0.05$.

a)



b)

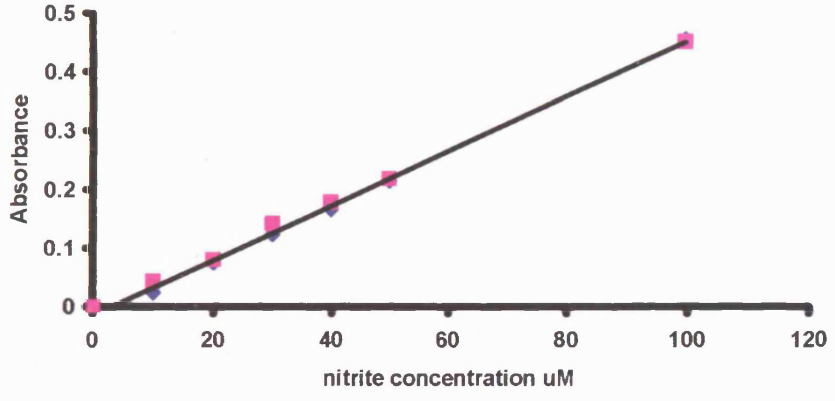


Figure 4.9. Greiss assay standard curve using media supplemeted with hemin and SnPPIX

The representative nitrite standard curves show the absorbance of both nitrite standards (♦) and the reduced nitrate standards (■) following reduction with nitrate reductase. In figure a) standards are made up in media supplemented with 20μM SnPPIX , in figure b) standards are made up with media supplemented with 100μM hemin.

the media alone standard curve, the media plus hemin and the media plus SnPPIX standard curve for nitrite is shown in **Figure 4.10**. This indicates that when 100μM hemin or 20μM SnPPIX was included in the media used to make up the standard curve for nitrite absorbance, values obtained were no different than when just media was used alone. Although the hemin and SnPPIX are coloured and do indeed cause a higher absorbance reading, once the zero concentration absorbance reading has been subtracted from all readings this standardised the curve.

Astrocytes were treated with 100μM hemin for 3,6,18 and 24 hours. Control astrocytes in 6-well plates were treated with media alone for the specified times. Astrocytes were also treated with 20μM SnPPIX alone or 100μM hemin plus 20μM SnPPIX. At the end of the treatment time the media was removed and assayed for nitrite via the Griess reaction after any nitrate had been converted to nitrite by nitrate reductase. Results were expressed against protein per well. Incubation with hemin for 3,6,18 and 24 hours did not have a significant effect on nitrate/nitrite levels in astrocytes as compared to controls (**Figure 4.11.**).

4.5. Discussion

In the astrocyte cell culture system used here, treatment of astrocytes with 100μM hemin did not cause significant cell death as assessed by LDH release which would indicate disrupted cell membranes and MTT assay which gives an indication of the redox capability of the cell. These are both assays routinely used

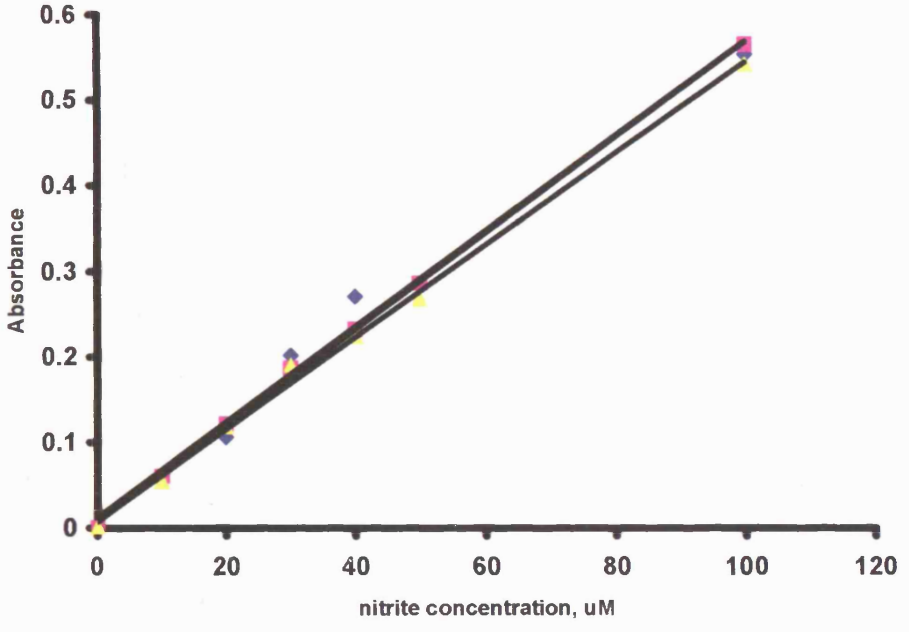
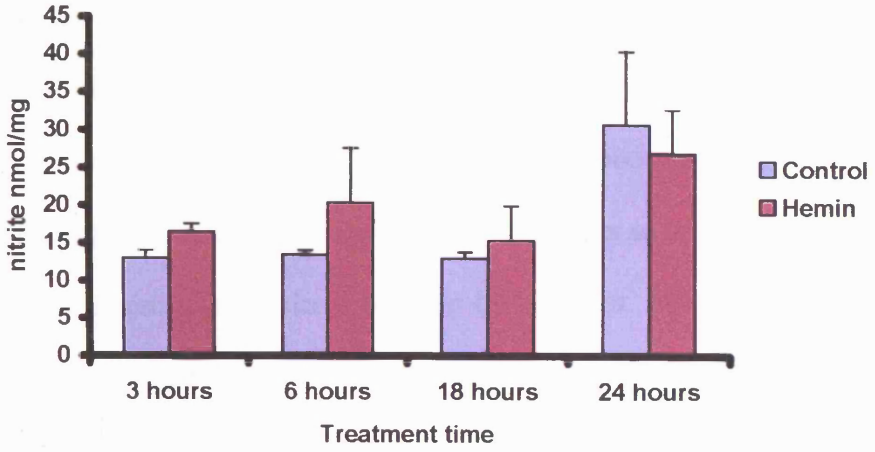


Figure 4.10. Comparison of nitrite standard curves

The standard curves shown are for nitrite in media alone (♦) nitrite in media + hemin (■) and nitrite in media + SnPPIX (▲).

a)



b)

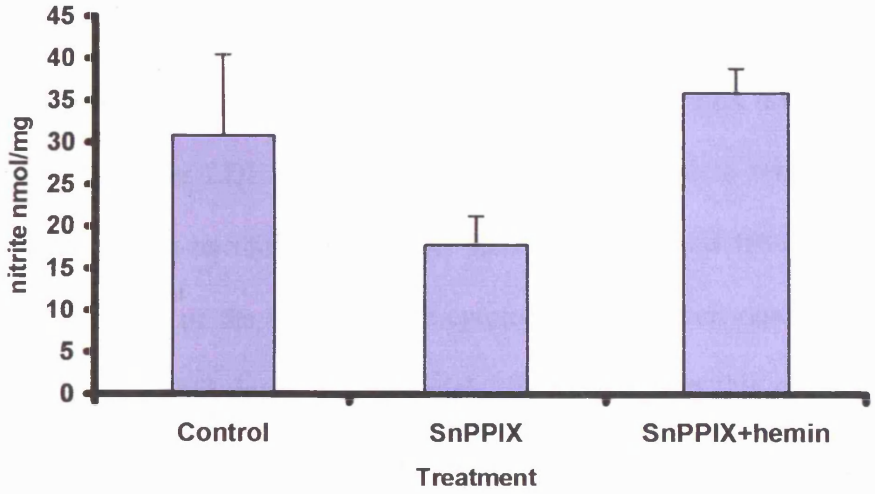


Figure 4.11. Nitrite/nitrate levels in media after astrocytes were treated with hemin over time and an HO inhibitor

Astrocytes were treated with medium only (control) or with 100μM hemin for 3 – 24 hours. Cells were also treated with 20μM SnPPIX alone or 20μM SnPPIX + 100μM hemin for 24 hours. Accumulated nitrite levels in the medium were measured. Hemin treatments for periods of up to 24 hours did not significantly effect nitrite levels as compared to controls. Treating with SnPPIX alone or hemin + SnPPIX also had no significant effect on nitrite production compared to 24 hour control. Data are mean ± SEM (n = 4 -6). Statistical analysis was by one-way ANOVA followed by least significant difference test.

to evaluate cell viability (Goldstein *et al.*, 2003;Regan *et al.*,2002; Koh & Choi, 1988) The decision to use 100μM hemin was after Clark *et al.*, (2000), with the intention of upregulating HO-1 activity as much as possible whilst maintaining cell viability. In their paper, Clark *et al.*, used concentrations of hemin up to 200μM to treat vascular smooth muscle cells in order to upregulate the activity of H0-1 and investigate cellular protection by bilirubin. It was discovered that the highest enzyme activity and hence amount of bilirubin production was achieved with a concentration of 100μM. At the 200μM concentration, activity was diminished and expression of inducible heat-shock protein 70 (hsp70) was stimulated, suggesting hemin toxicity.

The HO inhibitors tested gave very different results, SnPPIX did not affect cell viability by either LDH release or the ability of the cell to reduce MTT, but ZnPPIX caused a two-fold increase in LDH leakage and a 40% decrease in the redox capability of the cells. Similar cytotoxicity has been reported previously (Lutton *et al.*, 1997, Jozkowicz & Dulak, 2003), however this may be cell type dependent (Jozkowicz & Dulak, 2003). The central atom in haem analogues does appear to affect the biological properties of metalloporphyrins. Tin when incorporated into the porphyrin ring structure is extremely stable and there is at present no known physiological method by which it can be removed from the complex (Jozkowicz & Dulak 2003). Zinc porphyrins however are highly labile (Lutton *et al.*, 1997) and it has been postulated that their cytotoxicity may be due in part to the release of the zinc atom from the porphyrin ring, as zinc is able to substantially alter normal cell functions, leading to inhibition of iNOS activity (Sakaguchi *et al.*, 2002) and increasing cell death rate (Koh *et al.*, 1996; Lutton *et al.*, 1997; Kim *et al.*, 1999).

It has previously been reported that hemin is toxic to cells in culture at low concentrations (Balla *et al.*, 1992; Bhoite-Solomon *et al.*, 1993; Braverman *et al.*, 1995) however different cell types may have differing resistance to oxidative stress, and different levels of endogenous antioxidants.. Chen & Regan., (2004) show considerable toxicity to mouse cortical astrocytes using 30-60μM hemin, however in their investigation cells were treated in media which contained no serum. In the current investigation 10% FCS was used in all treatment media. It may be that when serum is used in the treatment medium the hemin is able to bind with a /some serum components and is prevented from accumulating in cell membranes (Shaklai, 1985) and causing lipid peroxidation . It has been shown that hemin is a very effective catalyst of lipid peroxidation chain reactions (Van den Berg *et al.*, 1988).

HO-1 expression in control astrocytes was detectable but not enhanced at any time during the 24 hours of incubation. This is in agreement with reports that under normal unstressed conditions the main isoform of HO expressed in the brain is HO-2 (Ewing & Maines, 1992). Hemin treatment for 3 hours results in a prominent increase in HO-1 protein and protein levels remained increased for the duration of treatment. Induction of expression agrees with previous reports, Clark *et al.*, (2000) and Maruhashi *et al.*, (2004) find increased HO-1 protein expression after 2 hours of hemin treatment in smooth vascular cells and ECV304 cells respectively. Clark *et al.*, (2000) see a maximal induction at 4 hours expression, at 16 hours this expression was reduced. This was not the case in this thesis, where expression continued to increase up to the 24 hour timepoint, however the experimental protocol was different in that this group

(Clark *et al.*, 2000) incubated cells with hemin for only 2 hours followed by incubation with medium alone. Their results suggest an initial response to hemin followed by a return to basal conditions, since the cells in the present study were exposed to hemin throughout the treatment time we could expect the results to be different. Similarly Maruhashi *et al.*, (2004) saw maximal expression at 8 hours but then a reduced expression at 24, this may reflect different cell type or culture conditions. SnPPIX caused an increased expression of HO-1 protein either alone or when used in conjunction with hemin, and at first thought this may seem suprising, but it has been found that although SnPPIX is a potent inhibitor of HO activity it is also an inducer of HO-1 protein. Eisenstein *et al.*, (1991) found that administration of SnPPIX to rats resulted in a 17-fold induction of HO-1 protein in liver although HO activity actually decreased by >90%.

Expression of iNOS protein was also investigated, since after hemin treatment astrocytes take on a more stellate, apparently activated morphology and iNOS expression is found when cells are activated (Loihl *et al.*, 1999). Neither control nor hemin treated cells showed iNOS expression, a positive control, murine macrophage cell line, (RAW264.7 lysate stimulated with LPS and γ-interferon) confirmed that the antibody was detecting iNOS protein, interestingly a strong HO-1 band was also detected in this lysate. Not surprisingly the measurement of nitrite in culture media after culture treatment showed no significant difference from controls. It does appear that nitrite/nitrate levels do rise during the treatment period, however this is not a significant rise either when results are evaluated as time matched controls or as a single group.

Activity of HO, which is the combined activity of all HO isoforms, is significantly enhanced in astrocytes after 6 hours of hemin treatment compared to controls, this is also when activity is maximal, and although activity is still significantly higher than that of controls at 18 and 24 hours it is significantly lower at 24 hours than at 6. This peak in activity at around 6 hours and subsequent decline in activity is in agreement with previous investigations. A significant increase in HO activity has been found in human macrophages and human glioma cells after a 5 hour incubation with hemin (Yoshida *et al.*, 1988), a similar increase was seen in pig alveolar macrophages with a peak at 5-7 hours and then a gradual decline thereafter (Shibahara *et al.*, 1978). A maximal activity at 6 hours and decline thereafter was also recorded in skeletal muscle cells (Vesely *et al.*, 1998). The HO assay is a measure of the HO activity present within the cell at a particular time, the reason that the activity actually decreases with a longer treatment time may be that as well as being an inducer of the enzyme hemin can also inhibit the enzyme. There is some evidence for this in skeletal muscle cells, where Vesely *et al.*, (1998) found that although HO activity declined with increasing hemin concentration there were no decreases in protein or mRNA levels indicating that loss of activity was not due to downregulation of gene expression . In this study the fact that protein expression was still elevated at 24 hours suggests that this could be an enzyme function effect rather than a downregulation of protein synthesis. Administration of SnPPIX as mentioned before caused an increased expression of HO-1 protein, however an increase in HO activity did not follow with levels lower than control. The hemin mediated induction of HO activity was completely abolished when hemin was used in conjuction with SnPPIX suggesting that this haem analogue

is effective as an inhibitor of HO activity, indeed it has been reported that SnPPIX has a binding affinity for the enzyme greatly exceeding that of the natural substrate (Drummond & Kappas, 1981).

Measurement of respiratory chain enzyme activities showed no significant differences in hemin treated cells as compared to controls at any treatment time. This was true when activities were expressed against a protein baseline or against citrate synthase activity. Complex activites can be expressed against citrate synthase to allow for differences in mitochondrial enrichment between cell cultures (Selak *et al.*, 2000). As discussed previously hemin has been shown to be an oxidant (Shaklai *et al.*, 1985; Leclerc *et al.*, 1988) and it might have been expected that hemin treatment would have a detrimental effect on electron transport activities, however HO-1 induction and activity shown to be significantly raised at the 6 hour treatment timepoint would mean that bilirubin would be present in the cell and could act as an antioxidant . As well as bilirubin, cellular levels of reduced glutathione (GSH) may be protective, and the cell GSH concentration has been implicated in protecting the enzymes of the electron transport chain from oxidative stress (Bolanos *et al.*, 1995,1996; Barker *et al.*, 1996). Using hemin and SnPPIX however caused a significant increase in complex I activity, this may be due to oxidative stress caused by hemin treatment. It has been previously shown that despite the fact that oxidative stress has been shown to inhibit ETC enzymes, complex I activity can actually be raised in response to a mild oxidative stress (Ceaser *et al.*, 2003; Vasquez *et al.*, 2001). This could have effects downstream, and eventually cause inhibition of enzymes due to overproduction of electrons from complex I overwhelming the

other complexes. When astrocytes are treated with hemin alone, HO activity is stimulated and bilirubin is able to exert its antioxidant effects, however when HO activity is inhibited there will be minimal bilirubin present and a rise in complex I could conceivably occur.

Studies have shown that glutathione levels and HO-1 expression appear to be linked as a concerted stress response in the cell. Depletion of GSH causes an upregulation of HO-1 protein (Ewing & Maines, 1993; Oguro *et al.*, 1996; Ryter & Choi 2005) and addition of GSH or its precursors causes a decrease in HO-1 expression and activity (Foresti *et al.*, 1997). It has been reported that in mammals, after liver and kidney, the highest levels of GSH are found in the brain (Thompson *et al.*, 1999; Liu & Choi, 2000; Liu, 2002). Within the brain there are variations in GSH levels from region to region (Kang *et al.*, 1999; Liu, 2002) and also between cell types (Sagara *et al.*, 1993; Makar *et al.*, 1994; Bolanos *et al.*, 1995). For example cortical astrocyes cultured alone as used in this study, have higher GSH levels than neurones from the the same region (Raps *et al.*, 1989; Bolanos *et al.*, 1995).

In the present study GSH levels were significantly raised at 18 hours of treatment, this is when HO-1 activity has started to decrease and may be a response to hemin mediated oxidative stress. It has been shown that oxidants can increase expression of the first enzyme in the GSH synthesis pathway, glutamate-cysteine ligase. (Tian *et al.*, 1997; Moellering *et al.*, 1998; Iwata-Ichikawa *et al.*, 1999; Gegg *et al.*, 2005). It may be that whilst HO-1 activity is maximal bilirubin production is sufficient to deal with the oxidant challenge caused by hemin treatment , and this is why respiratory chain enzyme activity is preserved. Once

HO-1 activity starts to decline it is possible that GSH production is stimulated to take over this role. If this is the case then you would expect to see an increase in GSH levels when HO activity is inhibited, however after 24 hours of exposure to hemin and SnPPIX GSH levels in astrocytes are not significantly raised compared to 24 hour controls. The reasons for this are not clear, though it is possible that induction of H0-1 protein alone is preventing this rise in this situation. Kaizu *et al.*, (2003) report that preconditioning with SnPPIX induces HO-1 protein but attenuates ischemia/reperfusion injury in the rat kidney, independently of HO enzyme activation.

4.6. Conclusions

Hemin treatment induces both H0-1 protein and enzyme activity in astrocytes. The reason for maximal enzyme activity 6 hours after treatment is not clear, but is in agreement with other studies (Shibahara *et al.*, 1978; Yoshida *et al.*, 1988; Vesely *et al.*, 1998). Importantly levels of HO activity are still significantly raised 24 hours after hemin treatment .Although hemin can cause toxicity (Balla *et al.*, 1992; Bhoite-Soloman *et al.*, 1993; Braverman *et al.*, 1995; da Silva *et al.*, 1996), at the concentration used in this investigation and in the cell type used this is not evident. This may be due to the increase in HO activity causing bilirubin to be present, since it has been reported that bilirubin is the most abundant endogenous antioxidant in mammalian tissues and accounts for the majority of antioxidant activity in human serum (Gopinathan *et al.*, 1994). Normal GSH levels in astrocytes may also be protective here as GSH is a major antioxidant in the brain (Meister, 1988; Makar *et al.*, 1994; Nakamura *et al.*, 1997). The rise in

GSH levels at 18 hours following a decrease in HO activity does suggest a synchronisation of antioxidant defenses maintaining the status quo within the cell.

Hemin does not activate or induce iNOS protein or deleteriously affect respiratory enzyme chain activites making it a suitable HO-1 inducer to use whilst manipulating the NO status of the cell which will be the focus of the next chapter. SnPPIX has proven to be an effective inhibitor of HO activity and does not affect cell viability, making it a useful tool to study further HO-1 induction. However it does appear to have an effect on complex I when used in conjunction with hemin which may be caused by the absence of the antioxidant bilirubin due to HO activity being inhibited. It is possible that other anti-oxidants are being depleted also and whilst it has been shown that depletion of glutathione upregulates complex I activity in rat astrocytes and C6 glioma cells (Vasquez *et al.*, 2001) it could be possible that this is true for other protective molecules. SnPPIX alone does not appear to affect any of the parameters measured in this study apart from an upregulation of HO protein which has been documented previously (Sardana & Kappas, 1987).

Chapter 5

HO/iNOS interactions in astrocytes; effects on GSH status and mitochondrial function

5.1. Introduction

Production of NO by inducible nitric oxide synthase (iNOS) has been associated with perturbed mitochondrial function in a number of neurodegenerative disorders (for review see Stewart & Heales 2003). It has also been shown that NO and NO related species can markedly increase expression of HO-1 (Motterlini *et al.*, 1996, Foresti *et al.*, 1997, Forresti & Motterlini, 1999, Motterlini *et al.*, 2000). Indeed increased HO-1 expression is reported to be upregulated in many diseases including those that cause neurodegeneration (Schipper, 2004).

Nitric oxide (NO) is a small diffusible highly reactive molecule. It is synthesised in mammals by the nitric oxide synthases (Kwon *et al.*, 1990; Leone *et al.*, 1991). There are generally accepted to be three isoforms of NOS, NOS I (nNOS), NOS II (iNOS), and NOS III (eNOS). NOS isoforms I and II are Ca^{2+} dependent and constitutive, whereas NOS II is Ca^{2+} independent and inducible. Numerous roles for NO have been identified, for example, neurotransmitter release, morphogenesis, synaptic plasticity and regulation of gene expression (Dawson & Dawson 1996). However as mentioned above inappropriate formation of NO is now thought to be an important source of toxicity in many neurodegenerative disorders. In glial cells such as astrocytes this toxicity can occur via activation of iNOS, the inducible NOS isoform.

Bolanos *et al.*, (1994) showed that treatment of primary astrocytes with lipopolysaccaride (LPS) + interferon gamma (IFNγ) for 18 hours caused a 25%

irreversible inhibition of cytochrome *c* oxidase activity (complex IV of the mitochondrial respiratory chain) compared to untreated controls. After 36 hours this inhibition had risen to 56% and succinate-cytochrome c reductase activity (Complex II-III) was inhibited by 35% compared to untreated controls. This inhibition could be prevented by the presence of NG-mono-methyl-L-arginine, an iNOS inhibitor.

As already shown in the previous chapter hemin can be used to upregulate HO-1 expression and HO activity in astrocytes, thus a combination of hemin and IFNγ/LPS treatment with the appropriate inhibitors could be used to study HO/iNOS interaction within the cell.

Aims

Given that HO-1 expression is known to be enhanced by activation of astrocytes with IFNγ/LPS (Motterlini *et al.*, 1996; Foresti *et al.*, 1997; Foresti & Motterlini, 1999; Motterlini *et al.*, 2000), the aims of this chapter are to examine the effects of IFNγ/LPS mediated iNOS activation on HO activity, and whether manipulation of the HO status of the cell can affect mitochondrial respiratory chain activities, GSH status and nitrite production after IFNγ/LPS treatment. In addition an exogenous source of NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium1,2diolate (DETA-NO) will be used to examine the differences between an endogenous source of NO involving activation of iNOS , and an exogenous source of NO, which does not involve the

iNOS pathway, on HO-1 expression and activity, ETC enzyme activities, nitrite/nitrate production and GSH status.

5.2. Methods

5.2.1. Cell culture

Primary rat astrocytes were cultured as described in section **2.2.** and treated as described in the experimental protocols section. All treatments were for 24 hours.

5.2.2. HO assay

Bilirubin production by astrocytes in control and treated cells was determined as described in section **3.2.3**. and used as an indication of HO activity.

5.2.3. Spectrophotometric enzyme assays

Complexes I, II+III and IV of the ETC and citrate synthase (CS) were assayed in control and treated astrocyte homogenates as described in section **2.3**.

5.2.4. Total nitrite/nitrate assay

Accumulation of the stable breakdown products of NO breakdown nitrite and nitrate was measured in media from control and treated astrocytes as described in

section **2.4.1**. by the Griess assay. Nitrate was first reduced to nitrite using nitrate reductase.

5.2.5. GSH quantification

GSH content of control and treated astrocytes was analysed by reverse-phase HPLC as described in section **2.6**

5.2.6. Western blots for HO-1

Expression of HO-1 and iNOS proteins in control and treated astrocytes was determined by subjecting samples to SDS-PAGE to separate the proteins, then transferring the proteins to a nitrocellulose membrane and using antibodies to HO-1 as described in section **2.5**. To confirm equal loading of protein in each lane an actin antibody was used.

5.2.7. Protein determination

Sample protein concentration was determined using the Lowry method as described in section **2.7**.

5.3. Experimental Protocols

Hemin and SnPPIX treatments

Hemin or SnPPIX were dissolved in a minimal amount of 0.1M NaOH and made up to a stock concentration of 20mM with astrocytic medium, pH was checked to ensure the buffering ability of the media was sufficient to buffer this concentration of NaOH (<0.1 pH unit difference). All treatments were for 24 hours.

IFNγ/LPS treatments

IFNγ was made up to a stock concentration in sterile HBSS/0.1% BSA. Treatment was 100U/ml. LPS was made up to a stock concentration in sterile HBSS and treatment was 1μg/ml.

Depletion of GSH

Astrocytes were treated with media supplemented with 0.5mM L-buthionine-S,R-sulfoximine (L-BSO), an inhibitor of glutamate-cysteine ligase (GCL) (Meister, 1991) for 24 hours in 6-well plates. Media was removed, cells washed twice with HBSS and media containing appropriate treatment replaced for a further 24 hours.

DETA-NO treatments

DETA-NO was dissolved in astrocytic media and treatments were 0.5mM. . In control experiments, media was supplemented with the same concentration of degraded DETA-NO. This had been previously incubated at 37°C for 7 days.

None of the treatments significantly increased LDH release compared to controls

5.4. Results

5.4.1. Nitrite/nitrate accumulation in media after IFNγ/LPS treatment.

Astrocytes were seeded onto 6 well plates and treated with media alone (control), media supplemented with 100U/ml IFNγ + 1μg/ml LPS which have been shown previously to activate iNOS and with it NO production (Bolanos *et al* 1994; Brown *et al.*, 1995) or media supplemented with IFNγ/ LPS + 0.5mM L-NIL a specific inhibitor of iNOS (Bryk & Wolff), for 24 hours. Media was removed and nitrite levels were determined after nitrate conversion to nitrite as described in section **2.4.1**. Treatment with IFNγ/LPS caused a significant, more than two-fold increase in nitrite accumulation (**Figure 5.1**) (control, 30.8 ± 9.7 nmol nitrite/mg protein; IFNγ/LPS, 76.4 ± 10.5 nmol nitrite/mg protein). If L-NIL was used with IFNγ/LPS the rise in nitrite accumulation was abolished (**Figure 5.1**).

5.4.2. ETC activity after IFNγ/LPS treatment

Astrocytes were seeded onto 6 well plates and treated with media alone (control), media supplemented with 100U/ml IFNγ + 1μg/ml LPS or media supplemented with 0.5mM L-NIL a specific inhibitor of iNOS, for 24 hours. ETC complex activities were then measured in cell homogenates. Complex I activity was significantly higher in IFNγ/LPS treated cells than control when assessed against citrate synthase activity (**Figure 5.2**) (vs CS: control, 0.20 ± 0.01; IFNγ/LPS, 0.35 ± 0.01, ($n = 3$-6) $p < 0.05$).This was not the case when activity was assessed against protein however a high SEM in this set of results may well be the reason for this. Complex IV activity in cells treated with IFNγ/LPS was significantly

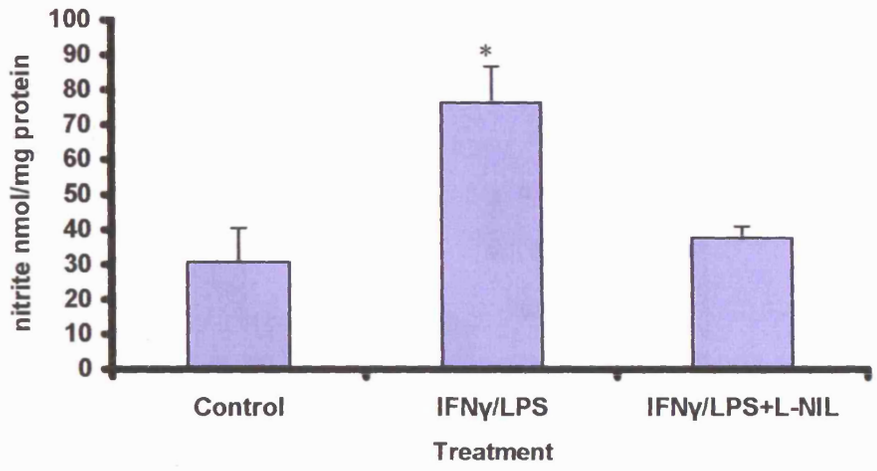


Figure 5.1. Nitrite/nitrate accumulation in media after IFNγ/LPS treatment
Astrocytes were treated with media alone (control), media supplemented with 100Uml IFNγ/ 1μg/ml LPS or supplemented with IFNγ/LPS + 0.5mM L-NIL, for 24 hours. Accumulation of nitrite in media of cells treated with IFNγ/LPS was significantly increased compared to controls. Use of the iNOS inhibitor L-NIL resulted in levels of nitrite accumulation not significantly different to controls. Data is mean ± SEM. Data was statistically evaluated by one-way ANOVA followed by LSD. *$p < 0.05$.

Treatment	Comp I nmol/min/mg	Comp II/III nmol/min/mg	Comp IV *k*/min/mg	Citrate Synth. nmol/min/mg
Control	32.2 ± 1.8	15.0 ± 2.3	2.12 ± 0.22	160.5 ± 9.5
IFNγ/LPS	54.9 ± 10	10.7 ± 1.4	0.55 ± 0.18*	141.0 ± 21.8
IFNγ/LPS+L-NIL	48.2 ± 6.9	11.5 ± 2.7	1.62 ± 0.19	163.1 ± 22.5

Table 5.1.

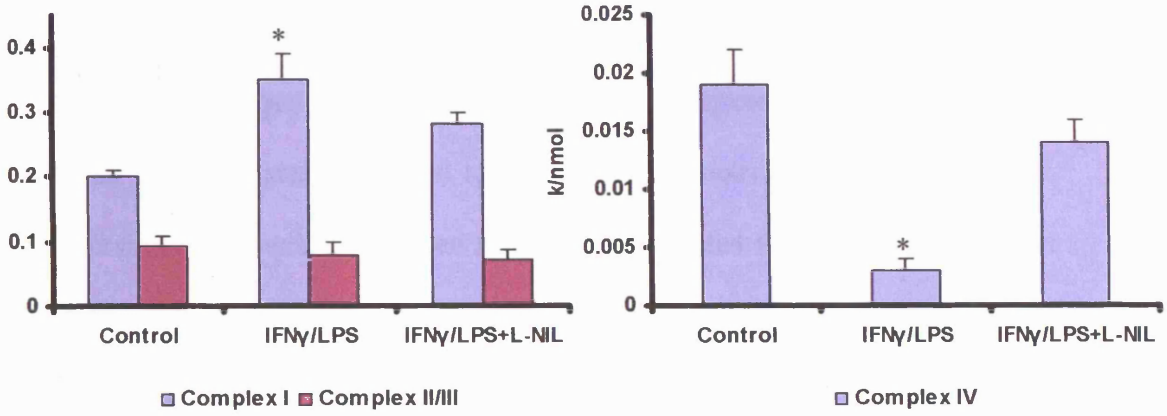


Figure 5.2. Effect of IFNγ/LPS treatment on the ETC

Astrocytes were treated with medium alone (control), medium supplemented with 100U/ml IFNγ + 1μg/ml LPS or medium supplemented with IFNγ/LPS + 0.5mM L-NIL. Following 24 hours of treatment cell homogenates were assayed for ETC complex activity. Data were expressed against protein and citrate synthase. IFNγ/LPS treatment caused a significant rise in complex I activity against control when measured against a citrate synthase baseline and a significant decrease in complex IV activity when measured against protein or citrate synthase . Data are mean ± SEM (n = 3-6) Data was statistically evaluated by one-way ANOVA followed by least significant difference test.* $p < 0.05$.

lower than controls whether assessed against a protein baseline or against citrate synthase activity (**Figure 5.2**) (vs protein: control, 2.12.± 0.22 nmol/min/mg protein; IFNγ/LPS, 0.55 ± 0.18 nmol/min/mg protein, (n = 3-6) $p < 0.05$). Stimulating astrocytes with IFNγ/LPS whilst using the iNOS inhibitor L-NIL gave ETC complex activites which were not significantly different to controls.

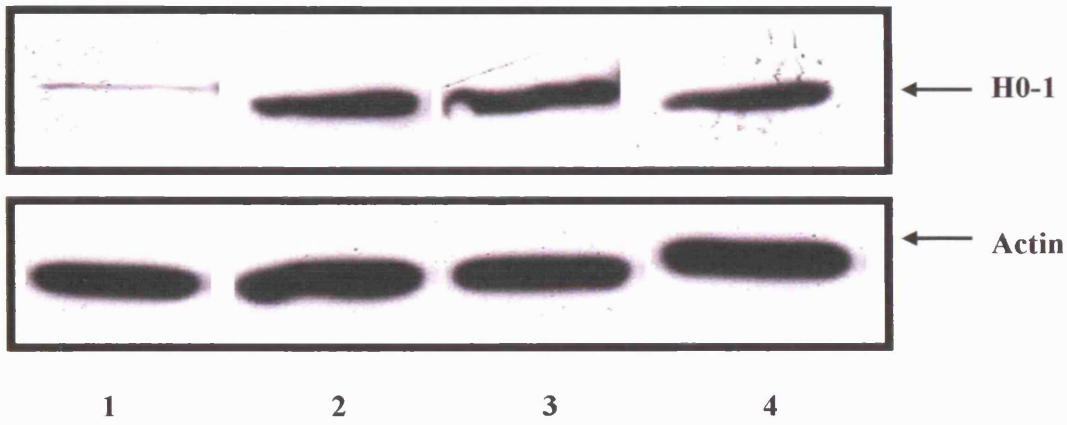
5.4.3. HO expression and activity after IFNγ/LPS treatment

Treatment of astrocytes with IFNγ/LPS causes enhanced expression of HO-1 protein which greater compared to expression in control cells (**Figure 5.3a**) , expression is still enhanced when astrocytes are treated with the iNOS inhibitor L-NIL, or the HO inhibitor SnPPIX (**Figure 5.3a**). IFNγ/LPS treatment caused a significant, approximately 2-fold increase in HO activity as compared to control activity (**Figure 5.3b**) (Control 21.3 ± 5.8 pmol bilirubin/min/mg protein; IFNγ/LPS 50.3 ± 3.6 pmol bilirubin/min/mg protein (n = 3-4 independent cell preparations) $p < 0.05$). However treatment of astrocytes with IFNγ/LPS and L-NIL or SnPPIX resulted in HO activity comparable to control levels despite raised expression.(**Figure 5.3b**).

5.4.4. ETC activities after IFNγ/LPS treatment and induction or inhibition of HO-1.

As previously mentioned treatment of astrocytes with IFNγ/LPS caused complex I to be significantly higher than control (assessed against a citrate synthase baseline) and complex IV to be significantly lower (assessed against protein or

a)



Key: 1 = Control, 2 = IFNγ/LPS, 3 = IFNγ/LPS + L-NIL, 4 = IFNγ/LPS + SnPPIX.

b)

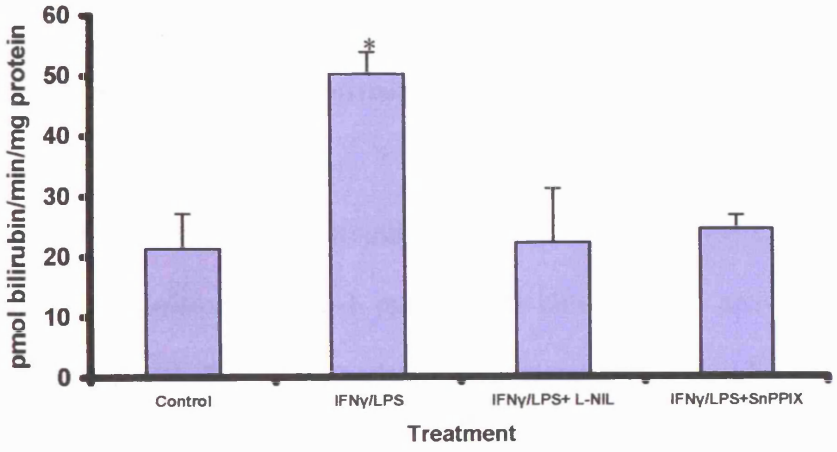


Figure 5. 3. The effects of IFNγ/LPS treatment on HO expression and activity

Flasks of astrocytes were treated with media alone (control), 100U/ml IFNγ/ 1μg/ml LPS with either 0.5mM L-NIL or 20μM SnPPIX. All treatments raised HO-1 protein expression against control. IFNγ/LPS treatment raised HO activity significantly vs control. Using an iNOS inhibitor (L-NIL) or an HO inhibitor with IFNγ/LPS brought activity back to control levels. Data are expressed mean ± SEM (n = 3-4). Data was statistically evaluated by one-way ANOVA followed by least significant difference test. *$p < 0.05$.

citrate synthase). Treatment with IFNγ/LPS + SnPPIX, the HO inhibitor, caused the activity of complex IV to be significantly decreased compared to control (**Table 5.2** and **Figure 5.4**) (vs protein: control , 2.12 ± 0.22 *k*/min/mg protein; IFNγ/LPS + SnPPIX, 1.0 ± 0.2 *k*/min/mg protein (n = 3 -5 independent cell preparations) $p < 0.05$). Treatment of astrocytes with IFNγ/LPS + hemin caused a significant rise in complex I activity when assessed against citrate synthase (**Figure 5.4**) (vs CS: control, 0.201± 0.01; IFNγ/LPS + hemin, 0.336 ± 0.07 (n = 3-5 independent cell preparations) $p < 0.05$). All other ETC complex activities including complex IV where treated with IFNγ/LPS + hemin , were not significantly different to controls.

5.4.5. Effect of inhibiting or inducing HO on HO activity and expression of HO-1 in IFNγ/LPS treated astrocytes

As discussed previously, treating astrocytes with IFNγ/LPS caused both enhanced expression of HO-1 protein and elevated HO activity. If astrocytes were treated with IFNγ/LPS and hemin, expression appeared to be greater than treatment with IFNγ/LPS alone (**Figure 5.5a**) and activity was significantly greater than control and IFNγ/LPS treated cells (**Figure 5.5b**) (control, 21.3 ± 5.8 pmol bilirubin/min/mg protein; IFNγ/LPS, 50.3 ± 3.6 pmol bilirubin/min/mg protein; IFNγ/LPS + hemin, 88.1 ± 7.1 pmol bilirubin/min/mg protein (n = 3-4 independent cell culture preparations) $p < 0.05$).

Treatment	Comp I nmol/min/mg	Comp II/III nmol/min/mg	Comp IV k/min/mg	Citrate synth. nmol/min/mg
Control	32.2 ± 1.8	15.0 ± 2.3	2.12 ± 0.22	160.5 ± 9.5
IFNγ/LPS+SnPPIX	46.7 ± 8.2	17.4 ± 2.5	1.0 ± 0.2*	199.7 ± 31.8
IFNγ/LPS+hemin	48.8 ± 9.7	15.1 ± 2.1	2.14 ± 0.45	151.5 ± 22.7

Table 5.2

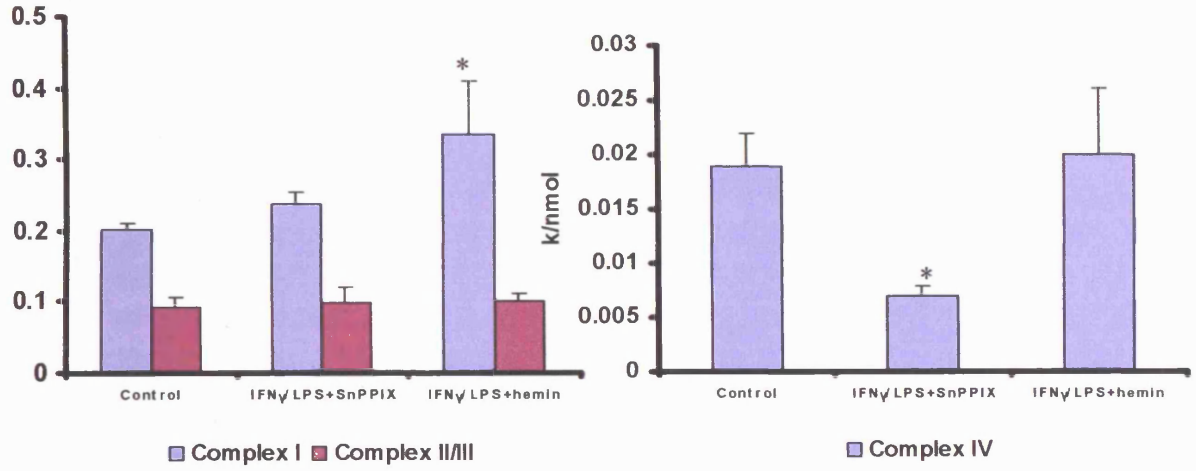
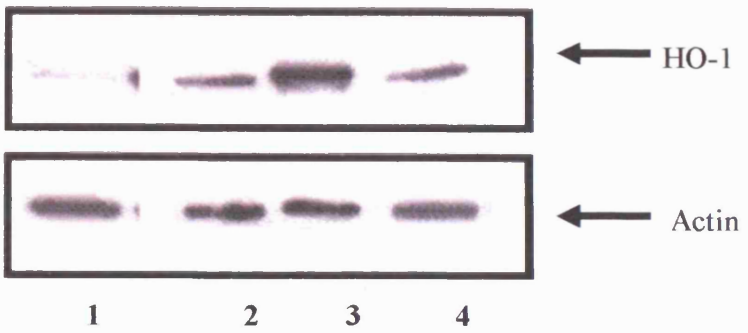


Figure 5.4. ETC enzyme activities after treatment with IFNγ/LPS and an inducer or inhibitor of HO.

Astrocytes were treated with media alone (control) or media supplemented with 100U/ml IFNγ/ 1μg/ml LPS + 20μM SnPPIX or IFNγ/LPS + 100μM hemin for 24 hours. Cell homogenates were assayed for ETC complex activity. Data were expressed against a protein baseline and citrate synthase activity. Treatment with IFNγ/LPS+hemin caused a significant rise in complex I activity against control when expressed against citrate synthase. A significant decrease in complex IV activity was seen when cells were treated with IFNγ/LPS+SnPPIX whether expressed against protein or citrate synthase. Data are mean ± SEM (n = 3-5 independent cell preparations). Statistical evaluation was by one-way ANOVA followed by least significant difference test. * $p < 0.05$.

a)



Key: 1 = Control, 2 = IFNγ/LPS, 3 = IFNγ/LPS + hemin, 4 = IFNγ/LPS + SnPPIX

b)

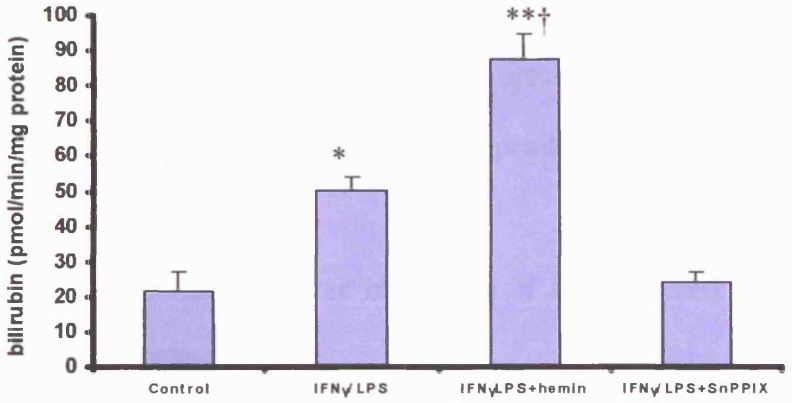


Figure 5.5. Expression and activity of HO when astrocytes are treated with IFNγ/LPS and inducers or inhibitors of HO-1

Astrocytes were treated with media alone (control) 100U/ml IFNγ/ 1μg/ml LPS, IFNγ/LPS + 100μM hemin or IFNγ/LPS + 20μM SnPPIX for 24 hours. Western blotting using an antibody to HO-1 (a) shows increased expression when cells are treated with IFNγ/LPS as compared to control, and expression is more enhanced when hemin is added to IFNγ/LPS. Activity of HO is raised significantly against control when astrocytes are treated with IFNγ/LPS (b) and significantly against control and IFNγ/LPS treated cells when hemin is added to IFNγ/LPS. HO activity data is mean ± SEM (n = 3-4 independent cell preparations). Statistical evaluation was by one-way ANOVA followed by least significant difference test. * $p < 0.05$, ** $p < 0.01$, †$p < 0.05$ against IFNγ/LPS treated cells.

5.4.6. Effect of inhibiting or inducing HO on nitrite/nitrate levels in media from astrocytes treated with IFNγ/LPS

Treatment of astrocytes with IFNγ/LPS causes iNOS to be activated and NO to be released by the cell, this significant NO production has been shown previously (**Figure 5.1.**). Adding hemin to IFNγ/LPS causes a significant increase in nitrite accumulation in media, (**Figure 5.6**) (control, 30.8 ± 9.7 nmol nitrite/mg protein; IFNγ/LPS + hemin 68.6 ± 10.1 nmol nitrite/mg protein (n = 3 -5 independent cell preparations) $p < 0.05$). Adding SnPPIX however to IFNγ/LPS causes nitrite accumulation in media to be even more significantly higher than control (**Figure 5.6**) (control, 30.8 ± 9.7 nmol nitrite/mg protein; IFNγ/LPS + SnPPIX, 100.9 ± 21.9 nmol nitrite/mg protein (n = 3-5 independent cell preparations) $p < 0.01$).

5.4.7. Effect of induction or inhibition of HO on GSH levels in astrocytes treated with IFNγ/LPS

Astrocytes treated with IFNγ/LPS + hemin showed significantly higer levels of glutathione than control cells or those treated with IFNγ/LPS alone (**Figure 5.7**) (control, 21.4 ± 3.6 nmol/mg protein: IFNγ/LPS, 19.2 ± 3 nmol/mg protein, IFNγ/LPS + hemin 50.8 ± 8.6 nmol/mg protein (n = 4 – 6 independent cell preparations) $p < 0.05$). Treatment with the HO inhibitor SnPPIX and IFNγ/LPS did not cause glutathione levels to change significantly.

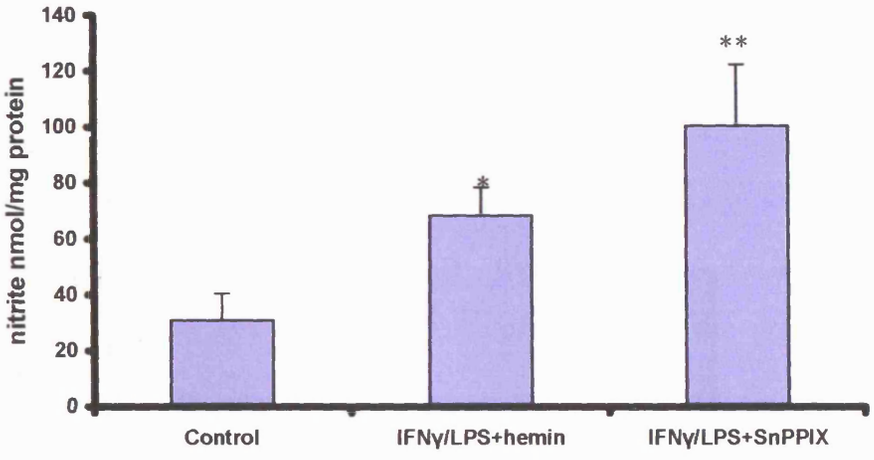


Figure 5.6. Nitrite/nitrate levels in media after treatment of astrocytes with IFNγ/LPS with an inducer or an inhibitor of HO-1.

Astrocytes were treated with media only (control) or with 100U/ml IFNγ/1μg/ml LPS + 100μM hemin or IFNγ/LPS + 20μM SnPPIX for 24 hours. Media was removed and nitrite levels were measured after nitrate was converted to nitrite as previously described. Treatment with IFNγ/LPS + hemin and IFNγ/LPS + SnPPIX caused nitrite levels to rise significantly compared to control. Data are mean ± SEM (n = 3-7 independent cell preparations). Statistical evaluation was by one-way ANOVA followed by least significant difference test.* $p < 0.05$ ** $p < 0.01$.

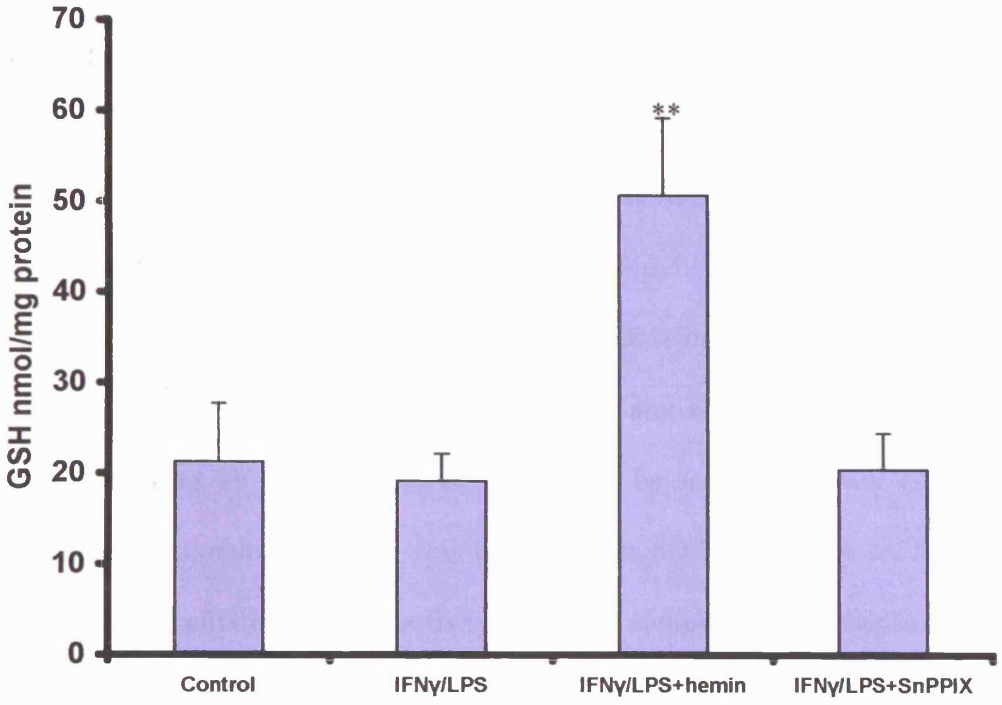


Figure 5.7. GSH levels in astrocytes treated with IFNγ/LPS and an inducer or inhibitor of HO.

Astrocytes were treated with media alone (control) or media supplemented with 100U/ml IFNγ/1μg/ml LPS + 20μM SnPPIX or IFNγ/LPS + 100μM hemin for 24 hours after which time cellular GSH levels were determined. Treatment with IFNγ/LPS + hemin caused levels to rise significantly compared to control. Other treatments had no significant effect. Data are mean ± SEM (n = 4-6 independent

cell preparations). Statistical evaluation was by one-way ANOVA followed by least significant difference test. ** $p < 0.01$.

5.4.8. Effect of simultaneously inducing and inhibiting HO on the ETC of IFNγ/LPS treated astrocytes

Since treating astrocytes with IFNγ/LPS + hemin appears to reverse the inhibition of complex IV seen after IFNγ/LPS treatment (**Table 5.2., Figure 5.4**.), it is feasible that induction of HO-1 by hemin is protective. Treating simultaneously with hemin and SnPPIX to inhibit HO may be expected to reverse this protective effect. Astrocytes were treated with media alone (control) or media supplemented with 100U/ml IFNγ/ 1μg/ml LPS + 100μM hemin + 20μM SnPPIX. When expressed against a protein baseline although there were no significant differences compared to control in any of the ETC complexes including complex IV all activities did appear to be somewhat lower (**Table 5.3.**). Although complex I activity was higher it was not significantly so. When assessed against citrate synthase activity however complex I was significantly higher than control (**Figure 5.8**) (vs citrate synthase: control; 0.201 ± 0.01, IFNγ/LPS+hemin+SnPPIX; 0.396 ± 0.01 (n = 3-4 independent cell preparations) $p < 0.05$)). No significant differences were seen in complex II/III or complex IV when expressed against citrate synthase.

5.4.9. Effect of simultaneously inducing and inhibiting HO on GSH levels in IFNγ/LPS treated astrocytes

In astrocytes treated with IFNγ/LPS + hemin GSH levels are more than two-fold higher than control levels (**Figure 5.7**.). To investigate GSH levels using this

Treatment	Comp I nmol/min/mg	Comp II/III nmol/min/mg	Comp IV *k*/min/mg	Citrate synth. nmol/min/mg
Control	32.2 ± 1.8	15.0 ± 2.3	2.12 ± 0.22	160.5 ± 9.5
IFNγ/LPS+hemin+SnPPIX	53.0 ± 3.6	9.9 ± 0.4	1.55 ± 0.21	137.9 ± 8.4

Table 5.3.

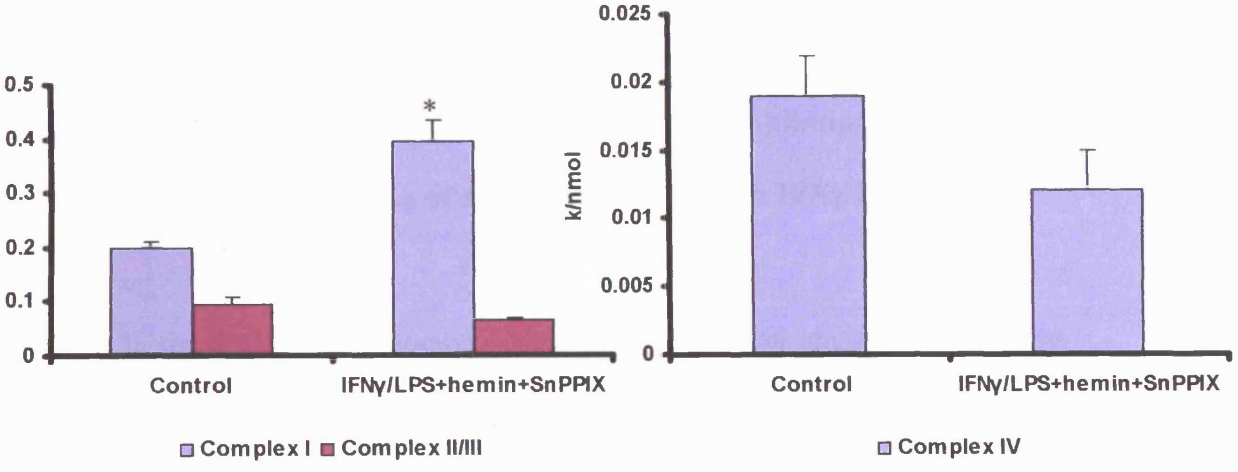


Figure 5.8. Effect of simultaneously stimulating and inhibiting HO on the ETC in IFNγ/LPS treated astrocytes

Astrocytes were treated with media alone (control) or media supplemented with 100U/ml IFNγ/ 1μg/ml LPS + 100μM hemin + 20μM SnPPIX for 24 hours, then ETC complex activities were assayed in cellular homogenates. Activities were expressed against a protein baseline or vs citrate synthase activity . There were no significant differences in activities when expressed against a protein baseline, however when expressed against citrate synthase there was a significant increase in complex I activity in cells treated with IFNγ/LPS+hemin+SnPPIX compared to control. Data was mean ± SEM (n = 3-4 independent cell preparations). Statistical significance was determined by one-way ANOVA followed by least significant difference test compared to control. * $p < 0.05$.

same treatment but with SnPPIX added to inhibit HO enzyme activity, cells were treated with media alone (control) or media supplemented with 100U/ml IFNγ/1μg/ml LPS + 100μM hemin + 20μM SnPPIX for 24 hours. Intracellular GSH levels in these cells were not significantly different from each other (**Figure 5.9**.) (control; 21.4 ± 3.6 nmol GSH/mg protein, IFNγ/LPS+hemin+SnPPIX, 23.0 ± 3.0 GSH nmol/mg protein (n = 4 -6 independent cell preparations)).

5.4.10. Effect of simultaneous induction and inhibition of HO on nitrite/nitrate levels in media of astrocytes treated with IFNγ/LPS

To see if simultaneously inducing and inhibiting HO had any effect on nitrite accumulation in the media of IFNγ/LPS treated astrocytes, cells were treated with media alone (control), media supplemented with 100U/ml IFNγ/1μg/ml LPS + 100μM hemin or IFNγ/LPS + hemin + 20μM SnPPIX for 24 hours. Nitrite levels were measured after any nitrate had been converted to nitrite (described in **section 2.4.1**.). Nitrite production in astrocytes treated with IFNγ/LPS + hemin or + hemin and SnPPIX showed nitrite significantly higher than control, but not significantly different from each other (**Figure 5.10**.) (control; 30.8 ± 9.7 nmol nitrite/mg protein, IFNγ/LPS+hemin; 68.6 ± 10.1 nmol nitrite/mg protein, IFNγ/LPS + hemin + SnPPIX; 79.1 ± 3.5 nmol nitrite/mg protein (n = 3 -5 independent cell preparations) $p < 0.05$).

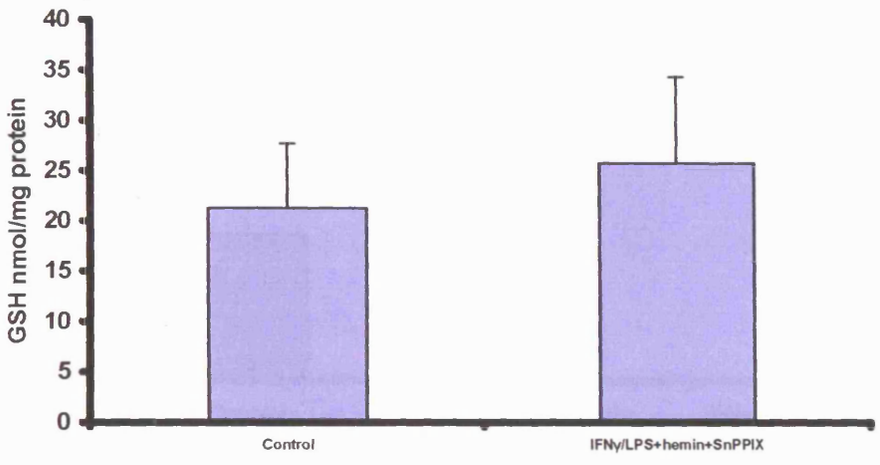


Figure 5.9. Effect of simultaneously inducing and inhibiting HO in astrocytes treated with IFNγ/LPS on GSH levels

Astrocytes were treated with media alone (control) or 100U/ml IFNγ/1μg/ml LPS + 100μM hemin + 20μM SnPPIX for 24 hours. Intracellular GSH levels were then determined. Treating with IFNγ/LPS + hemin + SnPPIX did not cause a significant change in the level of GSH compared to control. Values are mean ± SEM (n = 4-6 independent cell preparations). Statistical significance was determined by one-way ANOVA followed by least significant difference test.

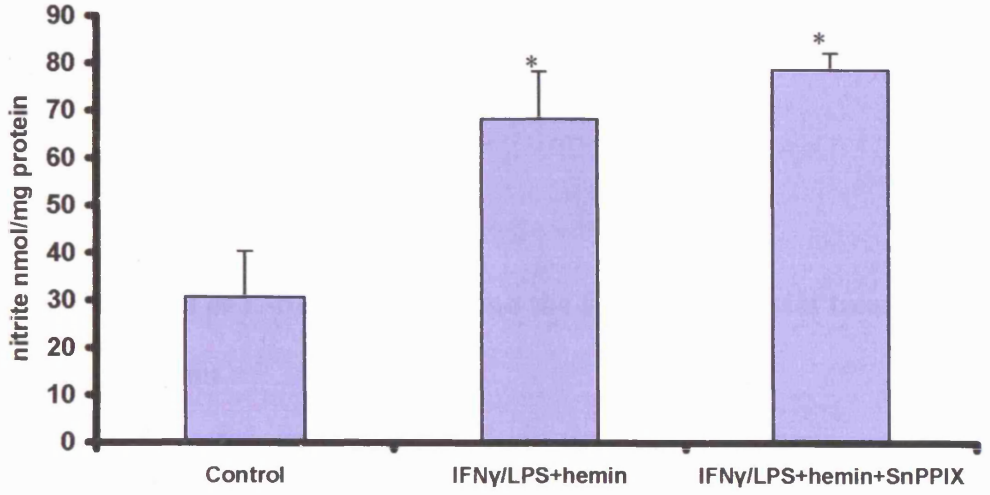


Figure 5.10. Effect of simultaneously inducing and inhibiting HO in astrocytes treated with IFNγ/LPS on nitrite/nitrate levels in media

Astrocytes were treated with media alone (control), 100U/ml IFNγ/1μg/ml LPS + 100μM hemin or IFNγ/LPS + hemin + 20μM SnPPIX for 24 hours. Media was removed and nitrite levels were measured after nitrate was converted to nitrite as described previously. Treatment with IFNγ/LPS + hemin and IFNγ/LPS + hemin + SnPPIX caused nitrite levels in media to be significantly raised compared to control. Data are mean ± SEM (n = 3 -5 independent cell preparations). Statistical evaluation was by one-way ANOVA followed by least significant difference test. * $p < 0.05$.

5.4.11. The effect of L-BSO treatment on GSH levels in astrocytes

In order to deplete GSH levels in astrocytes they were treated with L-BSO for 24 hours before L-BSO medium was removed, cells washed and media replaced with media alone (control), 100U/ml IFNγ/1μg/ml LPS, or IFNγ/LPS + 100μM hemin, for a further 24 hours. GSH levels were depleted significantly compared to non L-BSO treated astrocytes in all cases (**Figure 5.11**.).

5.4.12. The effect of L-BSO treatment on the ETC of astrocytes treated with IFNγ/LPS ± hemin

Cells depleted of GSH as discussed in the previous section, were treated with with media alone (control), 100U/ml IFNγ/1μg/ml LPS, or IFNγ/LPS + 100μM hemin for 24 hours before being assayed for ETC activity. When expressed against protein concentration or citrate synthase activityof the cells there were no significant differences in activities caused by any of the treatments (**Table 5.4**).

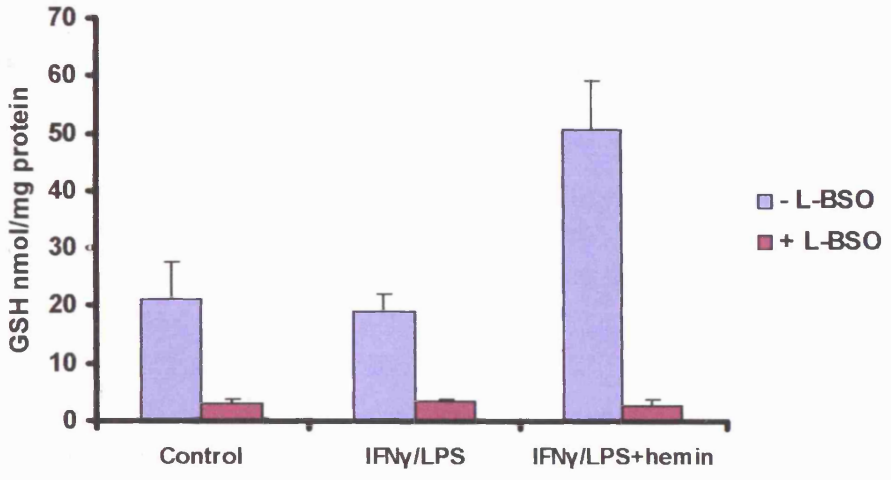


Figure 5.11. Intracellular GSH levels in astrocytes treated with IFNγ/LPS and hemin ± L-BSO

Astrocytes were treated with or without 0.5mM L-BSO for 24 hours after which time the medium was removed, cells washed twice with HBSS and incubated for a further 24 hours with media alone (control), media supplemented with 100U/ml IFNγ/1μg/ml LPS or IFNγ/LPS + 100μM hemin. Intracellular GSH levels were then determined. Treatment with L-BSO caused a significant drop in GSH levels in all cases compared to non L-BSO treated astrocytes (shown for comparison).

Treatment	Complex I nmol/min/mg	Complex II/III nmol/min/mg	Complex IV *k*/min/mg	Citrate synth. nmol/min/mg
Control	23.6 ± 4.1	11.8 ± 2.7	1.8 ± 0.2	117.5 ± 15.7
IFNγ/LPS	22.6 ± 2.9	11.5 ± 0.8	1.4 ± 0.3	105.1 ± 7.4
IFNγ/LPS+hemin	27.1 ± 7.1	13.2 ± 2.2	1.6 ± 0.2	90.8 ± 6.5

Table 5.4

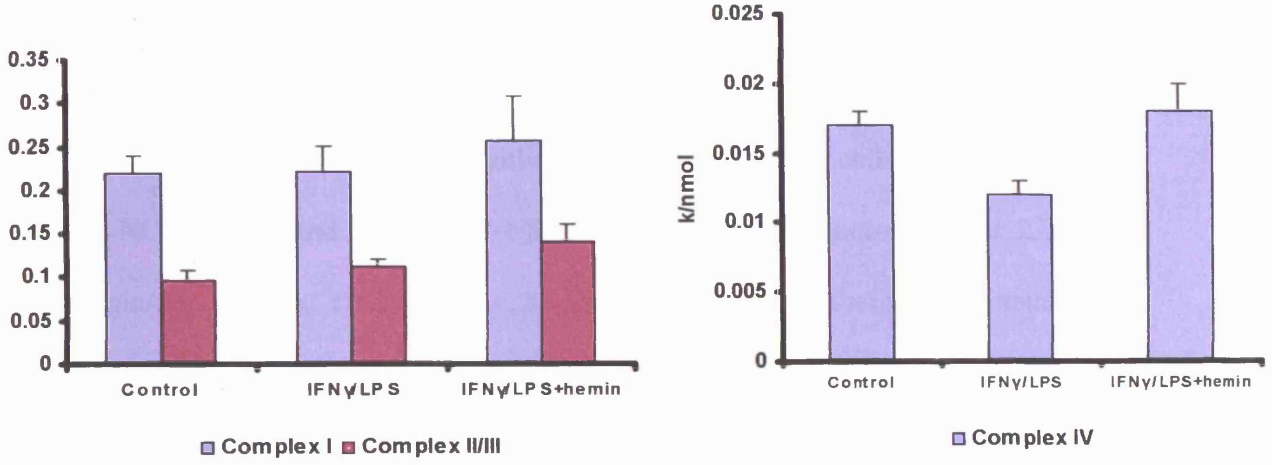


Figure 5.12. ETC complex activity of astrocytes after L-BSO treatment when astrocytes are treated with IFNγ/LPS and an inducer of HO

GSH depleted astrocytes were treated with media alone (control), or media supplemented with 100U/ml IFNγ/ 1μg/ml LPS or IFNγ/LPS + hemin for 24 hours before ETC complex activities were assayed in cellular homogenates. Activities were expressed against a protein baseline (Table 5.4) or vs citrate synthase activity (Figure 5.12). There were no significant differences in any complex activities when expressed against protein or citrate synthase activity.. Data was mean ± SEM (n = 4 independent cell preparations). Satistical significance was determined by one-way ANOVA followed by least significant difference test compared to control.

5.4.13. The effect of DETA-NO treatment on the astrocytic ETC treated with an inducer or inhibitor of HO

In order to investigate the effects of an exogenous NO exposure on astrocytes where the HO status of the cell is manipulated cells were treated with media alone (control), the NO donor DETA-NO (0.5mM), DETA-NO + 100μM hemin or DETA-NO + 20μM SnPPIX. ETC complex activities were measured and expressed against protein or citrate synthase activity. When expressed against protein, complex II/III was significantly lower than controls in cells treated with DETA-NO + hemin and DETA-NO + SnPPIX (**Table 5.5.**) (Control; 15.0 ± 2.3 nmol/min/mg protein, DETA-NO + hemin; 5.9 ± 0.6 nmol/min/mg protein, DETA-NO + SnPPIX; 6.5 ± 1.6 nmol/min/mg protein (n = 3 – 5 independent cell culture preparations) $p < 0.05$). When expressed against either protein or citrate synthase complex IV was significantly decreased in all treatments containing DETA-NO (**Table 5.5.** and **Figure 5.13**) (vs protein: control, 2.12 ± 0.22 k/min/mg protein; DETA-NO, 0.69 ± 0.14 *k*/min/mg protein; DETA-NO + hemin, 0.74 ± 0.06 k/min/mg protein; DETA-NO + SnPPIX, 0.49 ± 0.28 k/min/mg protein (n = 3 – 5 independent cell culture preparations) $p < 0.01$). Complex I and citrate synthase activities were not significantly different to controls after any of the treatments. Previous work in this lab has shown that degraded DETA-NO has no effect on respiratory chain enzyme activities (personal communication, Dr A Duncan, Institute of Neurology, London).

Treatment	Comp I nmol/min/mg	Comp II/III nmol/min/mg	Comp IV k/min/mg	C/Synthase nmol/min/mg
Control	32.2 ± 1.8	15.0 ± 2.3	2.12 ± 0.22	160.5 ± 9.5
DETA-NO	26.2 ± 8.4	8.3 ± 1.4*	0.70 ±0.15**	144.8 ± 10.7
DETA-NO+hemin	43.2 ± 11.2	5.9 ± 0.6*	0.74 ±0.06**	130.9 ± 9.3
DETA-NO+SnPPIX	27.3 ± 6.6	6.5 ± 1.6*	0.49 ±0.28**	110.5 ± 6.6

Table 5.5

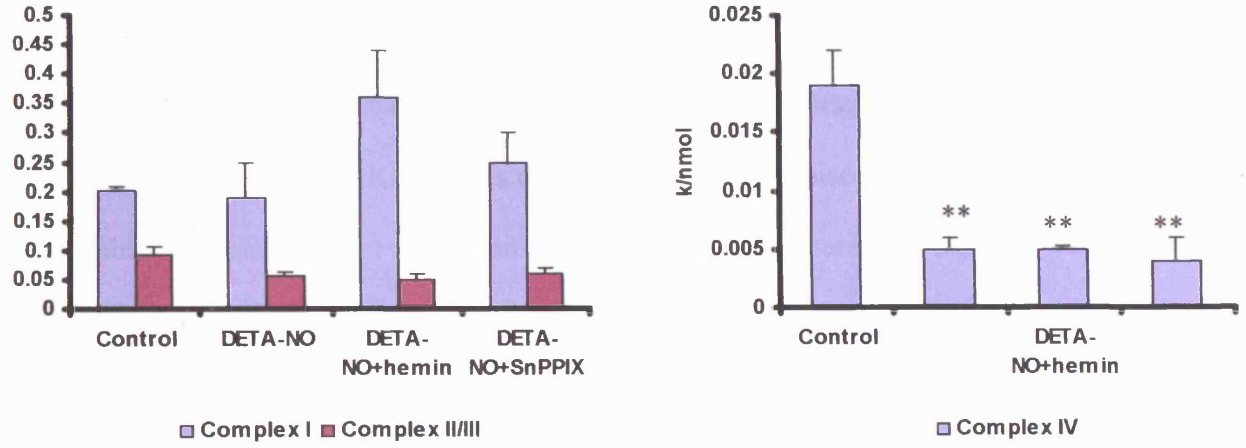


Figure 5.13. The effect of DETA-NO on the ETC when astrocytes are treated with an inducer or inhibitor of HO

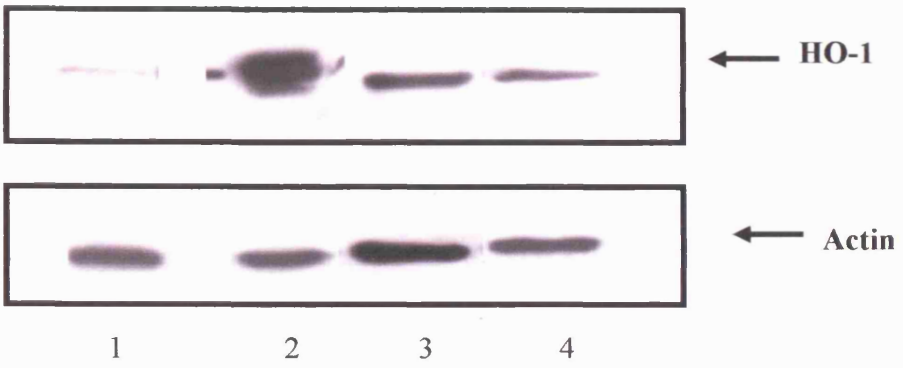
Astrocytes were treated with media alone (control), media supplemented with 0.5mM DETA-NO alone, 100μM hemin + DETA-NO or 20μM SnPPIX + DETA-NO. Following 24 hours of treatment cell homogenates were assayed for ETC complex activity. Data were expressed against protein and citrate synthase activity. Treatment with DETA-NO + hemin or SnPPIX caused a significant loss of complex II/III activity against control when assessed against protein. All DETA-NO treatments caused a significant loss of complex IV activity against a protein baseline or citrate synthase. Data are mean ± SEM (n = 3 -5). Data was statistically evaluated using one-way ANOVA followed by least significant difference test. * $p < 0.05$, ** $p < 0.01$.

5.4.14. The effect of DETA-NO treatment on HO-1 expression and HO activity

As shown in **Figure 5.5.** stimulation of NO production by iNOS mediated by IFNγ/LPS causes upregulation of HO-1 expression and HO activity. Would an exogenous source of NO have the same effect?

Astrocytes in flasks or 6-well plates were treated with media alone (control), media supplemented with 0.5mM DETA-NO, DETA-NO + 100μM hemin or DETA-NO + 20μM SnPPIX. Cells in 6-well plates were lysed and subjected to SDS-PAGE before being transferred to a nitrocellulose membrane which was probed with an antibody to HO-1. Cells in flasks were homogenised and assayed for bilirubin production as an indication of HO activity. Representative blots of HO-1 protein (**Figure 5.14a**) show that DETA-NO strongly induces expression of protein. DETA-NO + hemin or SnPPIX also appeared to raise expression of HO-1 compared to controls but not to the same extent. DETA-NO treatment alone caused a significant almost 4-fold increase in HO activity compared to control (**Figure 5.14b**) (Control; 21.3 ± 5.8 pmol biliubin/min/mg protein, DETA-NO; 75.1 ± 20.8 pmol bilirubin/min/mg protein (n = 3 -4 independent cell culture preparations) $p < 0.01$). In cells treated with DETA-NO + hemin the increase is still significant but smaller (**Figure 5.14b**) (Control; 21.3 ± 5.8 pmol bilirubin/min/mg protein, DETA-NO + hemin; 58.4 ± 9.6 pmol bilirubin/min/mg protein (n = 3 – 5 independent cell culture preparations) $p < 0.05$). Treating with SnPPIX inhibits HO activity as previously seen. Treating with degraded DETA-NO caused no rise in HO activity.

a)



Key: 1 = Control, 2 = DETA-NO, 3 = DETA-NO + hemin, 4 = DETA-NO + SnPPIX.

b)

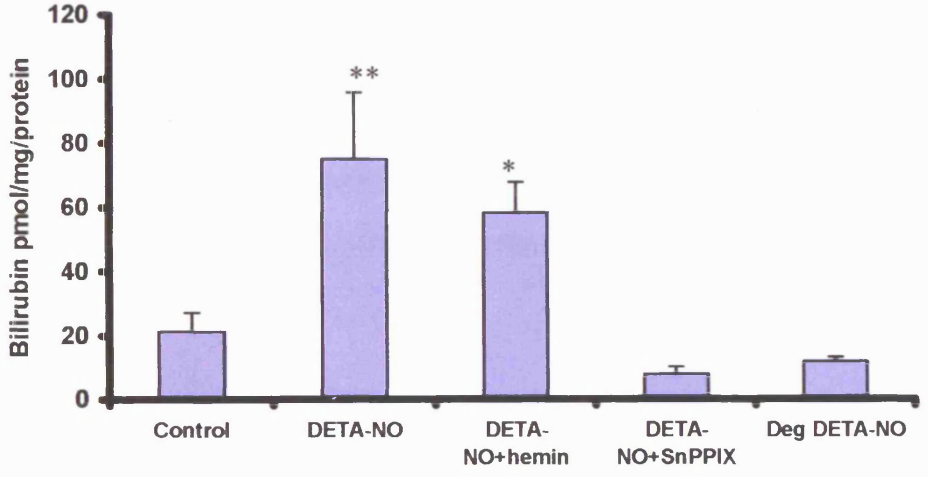


Figure 5.14. The effect of DETA-NO treatment on HO expression and activity in astrocytes

Astrocytes were treated with media alone (control), media supplemented with 0.5mM DETA-NO, DETA-NO+100μM hemin , DETA-NO+20μM SnPPIX or degraded DETA-NO for 24 hours. Bilirubin production was measured, and HO-1 expression was assessed by western blotting. Treating with DETA-NO increases expression of HO-1 protein alone or when combined with hemin or SnPPIX. HO activity is significantly increased compared to control when astrocytes are treated with DETA-NO alone or when treated with DETA-NO plus hemin. Data are mean ± SEM. Statistical evaluation was by one-way ANOVA followed by least significant difference test.*$p < 0.05$, ** $p < 0.01$.

5.4.15. Nitrite/nitrate accumulation in media after astrocytes treatment with DETA-NO whilst HO is induced or inhibited

In order to investigate whether there were any changes to the production of NO by DETA-NO when HO is induced or inhibited astroytes were treated with media alone (control) media supplemented with 0.5mM DETA-NO, DETA-NO + hemin or DETA-NO + SnPPIX for 24 hours. After this time media was removed, any nitrate converted to nitrite as described in **section 2.4.1.** and nitrite measured. All DETA-NO treatments caused significantly increased nitrite accumulation compared to control (**Figure 5.15**) (Control; 30.8 ± 9.7 nmol/mg protein, DETA-NO; 176.4 ± 33.8 nmol/mg protein, DETA-NO + hemin; 159.2 ± 17.5 nmol/mg protein, DETA-NO + SnPPIX; 197.9 ± 28.3 nmol/mg protein(n = 5 – 9 independent cell culture preparations) $p < 0.01$). None of the DETA-NO treatments however were significantly different from each other.

5.4.16. GSH levels in astrocytes treated with DETA-NO whilst HO is induced or inhibited

As described in section **5.3.6.** in astrocytes treated with IFNγ/LPS + hemin, levels of intracellular GSH rise significantly. In order to see if DETA-NO treatment has the same effect astrocytes were treated with media alone (control), 0.5mM DETA-NO, DETA-NO + 100μM hemin or DETA-NO + 20μM SnPPIX for 24 hours after which time GSH levels were measured . Treatment of cells with DETA-NO + hemin caused a significant, more than two-fold increase in

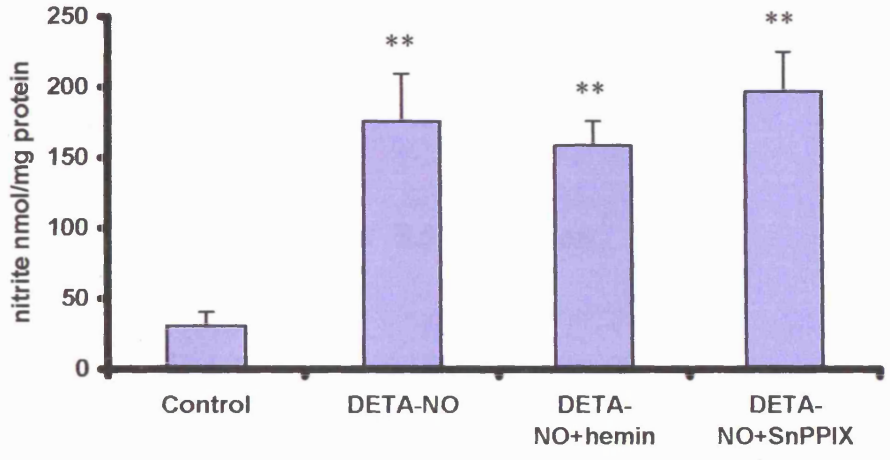


Figure 5.15. Nitrite/nitrate accumulation in media after treatment with DETA-NO and an inducer or an inhibitor of HO

Astrocytes were treated with media alone (control), media supplemented with 0.5mM DETA-NO alone, 100μM hemin + DETA-NO or 20μM SnPPIX + DETA-NO for 24 hours. Accumulated nitrite in media was determined after nitrate had been converted to nitrite as described previously. All treatments containing DETA-NO caused nitrite levels to be significantly raised compared to control. Data are mean ± SEM (n = 5 – 9 independent cell preparations). Data was statistically evaluated by one-way ANOVA followed by least significant difference test. ** $p < 0.01$.

intracellular GSH levels (**Figure 5.16**) (Control; 21.4 ± 3.6 nmol/mg protein, DETA-NO + hemin; 58.7 ± 7.7 nmol/mg protein (n = 4 – 6 independent cell culture preparations) $p < 0.01$). Treatment with DETA-NO alone or DETA-NO + SnPPIX did not significantly change GSH levels compared to control. Treatment of astrocytes with degraded DETA-NO does not result in any significant change in GSH levels as compared to untreated controls. (Gegg *et al.*, 2003).

5.5. Discussion

NO is rapidly converted to nitrate (NO_3^-) and nitrite (NO_2^-) after synthesis and release from astrocytes, therefore levels of these stable breakdown products in culture media from NO producing astrocytes is often used as an index of NO production (McNaught & Jenner, 2000a), and can be further postulated to be an index of iNOS activity .

Astrocytes in this study, when treated with interferon gamma (IFNγ) plus lipopolysaccaride (LPS) show stimulated iNOS activity as demonstrated by nitrite and nitrate release into media. Although IFNγ and LPS stimulate iNOS when used individually, Bolanos *et al*, (1994) showed that using both IFNγ and LPS potentiated the release of nitrite and nitrate as compared to release from astrocytes treated with either agent alone. Similarly it was found that incubating with both IFNγ plus LPS gave a 20-fold higher NOS activity than that found by Galea *et al*, (1992) after treatment with LPS alone. This agrees with the proposed idea that cytokines may mediate the induction of NOS activity by LPS (Murphy *et al.*, 1993, Simmons and Murphy, 1993).

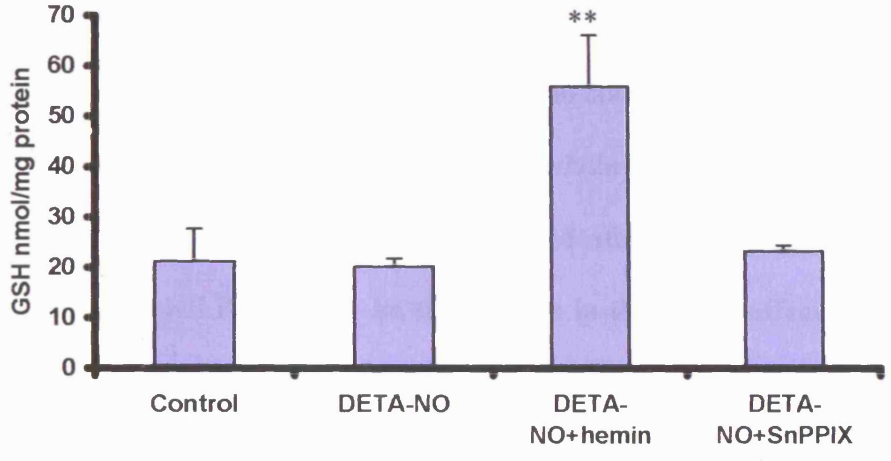


Figure 5.16. GSH levels in astrocytes treated with DETA-NO and an inhibitor or inducer of HO

Astrocytes were treated with media alone (control), media supplemented with 0.5mM DETA-NO, 100μM hemin + DETA-NO or 20μM SnPPIX + DETA-NO for 24 hours after which time cellular GSH levels were determined. Treatment with hemin + DETA-NO caused a significant rise in cellular GSH compared to control. Data are mean ± SEM (n = 4 – 6 independent cell preparations). Statistical evaluation was by one-way ANOVA followed by least significant difference test. ** $p < 0.01$.

Bolanos *et al* (1994) found that treating with IFNγ/LPS for 18 hours caused a 25% inhibition in the activity of complex IV; in the current study astrocytes were treated for 24 hours, and at this point a 75% inhibition was seen. Some of the increased inhibitory effects could be due to the longer incubation, although Bolanos *et al.*, (1994) found that treating the cells up to 36 hours in IFNγ/LPS only caused a 56% inhibition of complex IV. The discrepancy may be caused by a difference in treatment regime, where in the current study the treatment media was applied at time 0 and remained for the whole of the treatment time, whereas in Bolanos *et al* (1994) media was replaced after 18 hours with fresh media containing IFNγ/LPS. It may be that factors in the media affect the amount of inhibition seen. In this study it was also found that IFNγ/LPS treatment caused an apparent increase in complex I activity when assessed against a protein baseline, which became significant when assessed against citrate synthase activity. It has been reported that low grade oxidative stress can upregulate complex I activity in glial cells (Vasquez *et al.*, 2001) by increasing expression of the complex I ND6 subunit, a mitochondrially encoded subunit associated with proton translocation across the inner mitochondrial membrane. In this experimental situation oxidative stress could feasibly occur due to the inhibition of complex IV; Sipos *et al.*,(2003) report that in isolated nerve terminals production of ROS may occur when complex IV is more than 70% inhibited. Theoretically, once complex I acvtivity is upregulated then further electron leak could occur due to the inability of the other complexes to transport electrons quickly enough and therefore the situation could perpetuate itself.

It has been previously shown that treating various cells with IFNγ and /or LPS not only induces iNOS but also causes an upregulation of HO-1 protein, i.e. glial

cells (Kitamura *et al.*, 1998), glomerular mesangial cells (Datta *et al.*, 1999). However it is not clear how this upregulation relates to enzyme activity. Having established that in the astrocytes used in this study, treatment with IFNγ/LPS causes nitric oxide to be produced, inhibition of complex IV to occur and an increase in HO-1 protein, it was then necessary to determine if the HO protein was enzymatically active and investigate whether inhibition or further induction of HO would have any effect on the IFNγ/LPS mediated inhibition of complex IV.

Treatment with IFNγ/LPS caused an approximately two-fold upregulation of HO activity that was inhibited by treating with L-NIL, this suggests that the increase in activity is mediated by NO and not IFNγ/LPS treatment. This indicates that when complex IV is inhibited by IFNγ/LPS, HO and it's breakdown products will also be present. Reports have shown that inhibiting HO can in some cases be protective e.g. concurrent treatment of cultured astroglial cells with H_2O_2 and the HO inhibitor tin mesoporphyrin protected cells against the cytotoxic effects of H_2O_2 treatment (Dwyer *et al.*, 1988) and treatment of epithelial cells with an HO inhibitor partially reversed the toxic effects of a 24 hour H_2O_2 challenge (Da Silva *et al.*, 1996). In the present study cells treated with IFNγ/LPS and SnPPIX displayed a significant loss of complex IV activity when compared to controls but this inhibition was less than that seen when treating with IFNγ/LPS alone at approximately 43%, opposed to 75%. Treatment with hemin and IFNγ/LPS however, completely prevented the inhibition. Reasons for the apparent protection afforded by hemin can be postulated. HO activity measured after this treatment was significantly greater than that measured with IFNγ/LPS treatment

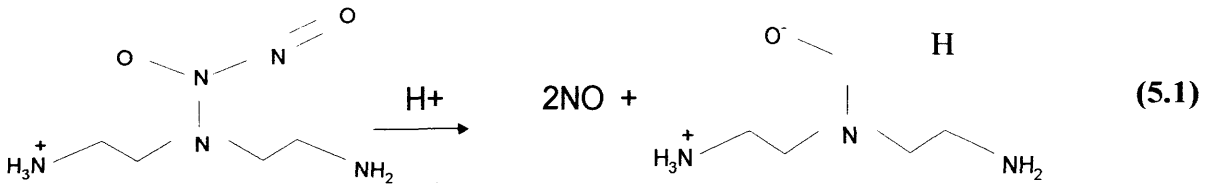
alone, it is a possibility that increased bilirubin production could have a powerful antioxidant effect. It is known that bilirubin is a scavenger of peroxy radicals such as peroxynitrite (Stocker *et al.*, 1987) which could be formed in this environment from NO and superoxide which may be present due to the apparent upregulation of complex I or inhibition of complex IV. It has also been observed that NOS also produces O_2^- and/or H_2O_2 in addition to NO (Heinzel *et al.*, 1992; Pou *et al.*, 1992). Expression of HO-1 after treatment with IFNγ/LPS plus hemin appears to be greater than that seen when treating with IFNγ/LPS alone or IFNγ/LPS plus SnPPIX and this could be an additional reason for the protective effect seen. Taylor *et al.*, (1998) report that actual induction of HO-1 protein rather than activity of the enzyme exerted protective effects against hyperoxic lung injury *in vivo*. In this case it may be expected that treatment with IFNγ/LPS plus SnPPIX would prevent the inhibition of complex IV, since SnPPIX has been shown to enhance synthesis of HO-1 protein whilst potently inhibiting enzyme activity (Sardana & Kappas, 1987), and it could be argued that inhibition is less in this instance, but not completely abolished. Examination of nitrite/nitrate accumulation in media show that adding hemin to IFNγ/LPS gives a similar result to using IFNγ/LPS alone, but adding SnPPIX to IFNγ/LPS causes an increased nitrite/nitrate production. Jozkowicz & Dulak (2003) report that in vascular smooth muscle cells SnPPIX increased IL-1β stimulated activity of iNOS and induced some nitrite accumulation in unstimulated cells. It is possible that in astrocytes stimulated with IFNγ/LPS, SnPPIX can further stimulate iNOS causing increased NO production, although media from astrocytes treated with SnPPIX alone did not show increased nitrite/nitrate production as shown in **Figure 4.11b**.

GSH levels were significantly higher in cells treated with IFNγ/LPS plus hemin compared to control cells or those treated with IFNγ/LPS plus SnPPIX. Since treatment of astrocytes with IFNγ/LPS alone does not raise GSH levels it may be that hemin is the cause since as mentioned previously it can act as an oxidant and oxidant stresses can cause upregulation of glutamate-cysteine ligase, the first and rate limiting enzyme in the GSH synthesis pathway (Tian *et al.*, 1997; Moellering *et al.*, 1998; Iwata-Ichikawa *et al* 1999; Gegg *et al.*,2005). Since GSH has been implicated in protecting the enzymes of the electron transport chain from oxidative stress (Bolanos *et al.*, 1995; Barker *et al.*, 1996) this may have some part to play in the prevention of inhibition of complex IV and the reason why treatment with SnPPIX is not protective to the same extent.

Complex activities, GSH levels and nitrite/nitrate accumulation were examined in IFNγ/LPS stimulated astrocytes treated with both hemin and SnPPIX to examine the effects of inducing and inhibiting HO simultaneously. In this case it appeared that complex II/III and IV were lower than controls, but this was not significant against either protein or citrate synthase activity. Complex I was significantly higher once more against citrate synthase and this could once again be explained by an oxidative stress caused by hemin. Nitrite/nitrate accumulation was not significantly different from that seen in astrocytes treated with IFNγ/LPS alone or IFNγ/LPS plus hemin. Perhaps surprisingly, GSH levels were not significantly different to control levels. As mentioned previously GSH levels and HO-1 expression appear to be linked as a concerted stress response in the cell, with depletion of GSH leading to an upregulation of HO-1 protein (Ewing & Maines, 1993; Oguru *et al.*, 1996; Ryter & Choi 2005) and addition of GSH and

its precursors causing a decrease in expression and activity (Foresti *et al.*, 1997). It may be expected that inhibiting HO activity would lead to an increase in GSH levels, however this may not be the case since the HO reaction itself can potentially generate hydrogen peroxide (H_2O_2), as a significant metabolic side product, as demonstrated by Noguchi *et al.*, (1983) Thus HO activity is a possible source of $OH^{\bullet}$ because both H_2O_2 and Fe^{2+} are released in the same microenvironment. Hence by inhibiting HO in this case a potential source of ROS is removed. Given the evidence obtained so far it could be possible that when astrocytes are treated with IFNγ/LPS alone, although HO-1 activity is induced, any oxidative stress arising is not enough to stimulate further GSH synthesis and so no further protection is afforded and complex IV is inhibited. However treatment with hemin and IFNγ/LPS causes further HO activity to be stimulated and together with the possible oxidative nature of hemin a threshold is reached whereby GSH synthesis is stimulated and protection occurs. An attempt to verify the protective nature of GSH was made by depleting astrocytic glutathione and treating with IFNγ/LPS ± hemin, to see if adding hemin was still protective. The depletion of glutathione was effective with depletion of >80% GSH, however the measurement of ETC activities on GSH depleted astrocytes shows no significant differences to depleted control astrocytes, whatever treatment was given. All complex activities were slightly lower than undepleted astrocytes but the only significant differences were in complex I where both IFNγ/LPS treated and IFNγ/LPS + hemin GSH depleted astrocytes showed significantly lower complex I values against a protein baseline, than their undepleted counterparts. Within the group differences caused by treatments were not found. Unfortunately in this case media was not analysed for nitrite/nitrate to

ensure that IFNγ/LPS were active and stimulating iNOS, if NO were not being formed it could explain the lack of inhibition of complex IV. Another possibility is that depleting GSH causes another protective mechansim to come into effect, indeed depletion of GSH has been shown to upregulate HO-1 (Ewing & Maines, 1993; Oguru *et al.*, 1996; Ryter & Choi 2005). Western blot analysis of IFNγ/LPS treated GSH depleted astrocytes could be carried out to show if this is the case. Other studies have shown that depleting astrocytic GSH in glial/neuronal co-culture studies reduces neuronal viability whilst leaving astrocytes largely unaffected (Drukarch *et al.*, 1997, Gegg *et al.*, 2005). Stimulation of iNOS with IFNγ/LPS causes endogenous release of NO within the cell and the subsequent inhibition of complex IV discussed above. DETA-NO is a nitric oxide donor which can provide an exogenous source of NO to cells in culture and given the apparent protective effect of treating IFNγ/LPS activated astrocytes with hemin it was considered conceivable that this same protection could be seen against an exogenous source of NO. DETA-NO when suspended in aqueous solution releases NO at a constant rate (**reaction 5.1**) with a half-life of at least 24 hours (Keefer *et al.*, 1996).



(5.1)

Previous work in this lab (Gegg, 2003) has shown that 0.5mM DETA-NO in astrocyte medium generates a steady state NO concentration of 0.93 ± 0.07 μM at 37°C. This was observed within 30 minutes of the DETA-NO being prepared and was constant for at least 24 hours.

The effect of 0.5mM DETA-NO on the electron transport chain was similar to that of IFNγ/LPS treatment in that complex IV was significantly inhibited compared to control, but also in this case complex II/III was inhibited too. In this case treating with hemin at the same time did not give any protective effects, in fact all treatments containing DETA-NO significantly lowered complex II/III against protein and complex IV against either variable. HO expression and activity were both present, in fact DETA-NO alone appeared to induce HO-1 protein to a much greater extent than when combined with either hemin or SnPPIX. This is contrary to reports that NO inhibits HO activity (Willis *et al.*, 1995; Juckett *et al.*, 1998,) and that NO donors prevent induction of HO-1 by hemin (Juckett *et al.*, 1998). Indeed although it appears that in the current study HO activity is lower when DETA-NO plus hemin are incubated with astrocytes there is not a significant difference and activity is still more than twice that of controls. Different NO-donors were used in the Willis paper which may account for some differences, also brain homogenate was used rather than cultured cells; in the Juckett paper, DETA-NO was used but the hemin incubation was 10μM for 1 hour before media was replaced with either untreated media or media containing DETA-NO. It may be significant that incubation of hemin and DETA-NO was not simultaneous, it may also be significant that the cells used were endothelial cells not astrocytes. Conversely, Foresti *et al*, (2003) report an

increase in HO activity when treating endothelial cells with DETA-NO that is further increased when hemin is also used. However in the present study this is not seen. The experimental protocols differ though and cell type so this could conceivably explain the differences. Nitrite/nitrate accumulation in media from DETA-NO treated astrocytes shows much higher levels than that seen when iNOS is stimulated, however this is not unexpected since in this situation all of the NO is released directly into the medium, not via cell membranes or intracellular targets such as the ETC or GSH as would be the case with iNOS generated NO. Also as mentioned previously a detectable release of 1μM NO is seen after 30 minutes of addition of DETA-NO to media (Gegg, 2003) so it is likely that NO release is occurring from very early on in the incubation, iNOS stimulation and subsequent release of NO is likely to take longer. Taking these factors into account, higher levels of NO may be expected to be available to inhibit the ETC more quickly, when treating with an NO-donor, and therefore a possible cause for the increased ETC inhibition seen in DETA-NO treated astrocytes after the same period of treatment as the IFNγ/LPS treated cells. It could possibly be expected, that if inhibition of the ETC is occurring via nitrosative stress, then GSH levels could be raised as GSH synthesis is stimulated; however, only hemin plus DETA-NO caused a significant rise in GSH levels. DETA-NO alone or with SnPPIX levels were similar to control. Despite this raise in GSH levels hemin does not apparently prevent inhibition of the ETC in astrocytes when used in conjunction with DETA-NO treatment. Since it has been suggested a rise in GSH levels acts as a protective mechanism when IFNγ/LPS stimulated astrocytes are treated with hemin it is not clear why it is not so in this case. A possible answer may be that NO released by DETA-NO reacts

very quickly with bilirubin and depletes it, thus although GSH levels have risen antioxidant defenses are lowered due to lower levels of bilirubin. Kaur *et al.*, (2003) show that various nitric oxide releasing species are able to interact with and decompose bilirubin very quickly. Kaur *et al.*, (2003) report that incubating 40μM DEA-NO with 5μM bilirubin caused the disappearance of the bilirubin within 90 minutes. Further to this Foresti *et al.*, (2003) report that haem degradation products appear to modulate the action of NO donors by accelerating and increasing the release of NO. Treatment of cells for 24 hours with hemin would certainly mean that haem degradation products would be present, however amounts of nitrite and nitrate were not significantly higher in DETA-NO treated cells as apposed to DETA-NO only treated cells. Another reason could be a delay in release of NO via iNOS in IFNγ/LPS treated cells which is not seen (or certainly not seen as much) when DETA-NO is used as the NO "donor". In order to see if this is this case it would be interesting to look at a time course of complex IV inhibition using both IFNγ/LPS and DETA-NO. Bolanos *et al.*, (1994) report that complex IV inhibition occurs in astrocytes after 18 hours of IFNγ/LPS treatment and previous work in this lab (Gegg, 2003) has shown that DETA-NO treatment also inhibits complex IV after 18 hours of treatment, but perhaps there is a timepoint before this when inhibiton is seen with DETA-NO treatment but not activation of iNOS.

5.6. Conclusions

Both an endogenous source (iNOS), and an exogenous source of NO cause inhibition of the ETC in astrocytes. Simultaneous stimulation of iNOS plus

hemin appear to reverse this inhibition, however simultaneous incubation of DETA-NO treated astrocytes with hemin does not. A number of factors may be involved in this apparent hemin mediated protection of the ETC. Upregulation of HO-1 protein and HO activity may be one factor. Treatment of astrocytes with hemin causes HO-1 to be induced and may also cause an oxidative stress due to the oxidative nature of the hemin molecule, this oxidative stress along with the potential H_2O_2 generating properties of the HO reaction may cause upregulation of GSH synthesis when a certain threshold is reached, thereby proving protective. When DETA-NO is used instead of stimulation of iNOS it may be that although there is HO induction and activity, rapid NO release from DETA-NO quickly depletes bilirubin , so a greater nitrosative stress is incurred. Although GSH is stimulated through the same pathway mentioned previously, the defenses may not be robust enough to prevent damage from occurring.

Chapter 6

General Discussion and Conclusions

Previous studies have documented increased HO-1 expression in many neurodegenerative disorders (for review see Schipper, 2004). It has also been documented that HO-1 expression is upregulated to high levels in glial cell populations as a response to oxidative stress (Ewing & Maines, 1991; Ewing *et al.*, 1992; Ewing & Maines., 1993; Geddes *et al.*, 1996; Turner *et al.*, 1998). Oxidative stress as a cause/consequence of neurodegeneration is a subject being researched by many different groups. This together with the increasing awareness of the importance of astrocytes in providing support to neurones and the fact that investigations to date on actual HO activity in astrocytes are few, makes the study of HO-1 expression and activity in astrocytes in reponse to oxidative and nitrosative stress important. Although HO-1 expression in astrocytes has been reported by various groups (Schipper, 1999; Ham & Schipper, 2000; Bidmon *et al.*, 2001; Lu & Ong, 2001; Mehindate *et al.*, 2001) a link to activity has been investigated far less frequently (Scapagnini *et al.*, 2002., Huang *et al.*, 2005).

Chapter 3 focused on the assay of haem oxygenase, and it was clear that there were certain steps in the assay process that are crucial. The liver cytosol extract used as a source of biliverdin reductase in the assay needed to contain as little contaminating blood as possible, to prevent haemoglobin interference in the assay, as reported by McNally *et al.*, (2004). Although many published works quote incubation times for the assay as an hour, in this investigation using astrocytes, activity was only linear up to 15 minutes of incubation (**Figure 3.3**), so this was the time scale used. Although as mentioned there are other methods for assaying HO activity, this is a method that does not require radioactive reagents or lengthy preparatory proceedures. The disadvantage of all the methods

of assaying HO activity is that it is total activity, the combined activity of HO-1 and HO-2 (and of course any other isoforms not identified yet) that are measured . The fact that HO-2 is constitutive (Maines, 1997) means that there will always be some contribution from this isozyme, but the fact that the only known chemical inducers of HO-2 are adrenal glucocorticoids suggest that any increase seen in HO activity when not using these agents is likely to be due to HO-1. Further, it has also been reported that HO-2 in the brain is mainly expressed in neurones (Ewing & Maines, 1992), so it may well be that the majority of HO activity seen in astrocytes is HO-1. Antibodies have been raised against both HO isoforms (Maines *et al.*, 1996; Trakshel *et al.*, 1996) which are reported not to cross react so it may be possible to immunoprecipitate each isoform from a cell culture homogenate and examine the contribution via an appropriate assay.

Hemin was used as both an inducer of HO-1 and as a substrate in the HO assay, and the fourth chapter of this thesis concentrated on the effects that hemin had on cell viability, total HO activity and HO-1 expression, mitochondrial ETC enzyme activity, GSH status and nitrite/nitrate production in rat astrocytes. Hemin can be a source of cell injury to glial cells within the brain due to haemorrhage, when haemoglobin spontaneously, nonenzymatically converts to methaemoglobin (Huang *et al.*, 2002) from which hemin readily dissociates (Bunn & Jandl., 1968; Hebbel & Eaton., 1989). The results of this chapter showed no significant loss of cell viability using 100μM hemin, but a robust upregulation of HO-1 protein and a corresponding increase in total HO activity (**Figure 4.6**.). Although HO activity was maximal at 6 hours of treatment there was still an approximately 3.5 fold increase in activity after 24 hours of treatment. All other parameters measured showed no significant differences

against control except for an increase in the levels of astrocytic GSH at 18 hours of hemin treatment and as mentioned previously this could be due to a decreased HO activity causing a transient oxidative stress which increases GSH synthesis, which is known to be upregulated by oxidative stress (Tian *et al.*, 1997; Moellering *et al.*, 1998; Iwata-Ichikawa *et al.*, 1999; Gegg *et al.*, 2005). This chapter showed that after 24 hours of hemin treatment, apart from a change in HO activity and expression all other measured parameters were not significantly different to control. The inhibition of HO activity was investigated using two metalloporphyrins, ZnPPIX and SnPPIX. ZnPPIX was discarded due to apparent toxic effects at the concentration used. Inhibition of HO activity was successfully achieved by using SnPPIX, which had no other effects on astrocytes apart from causing an increased complex I activity when used in conjunction with hemin, and an upregulation of HO-1 protein. Use of metalloporphyrin inhibitors has been a common feature of HO research, however recent reports have suggested results achieved using them should be viewed with some caution due to effects they may have on the cell unrelated to HO inhibiton (Jozkowicz & Dulak, 2003). It has been reported that SnPPIX alone can increase basal levels of nitrite/nitrate accumulation (Jozkowicz & Dulak, 2003), that was not the case seen in the current study and it may well be that responses are cell type specific and each case needs to be examined on its merits. Nonetheless other methods of preventing HO activity may be able to remove possible non-specific actions and they should be investigated where possible and will be discussed in the future work section.

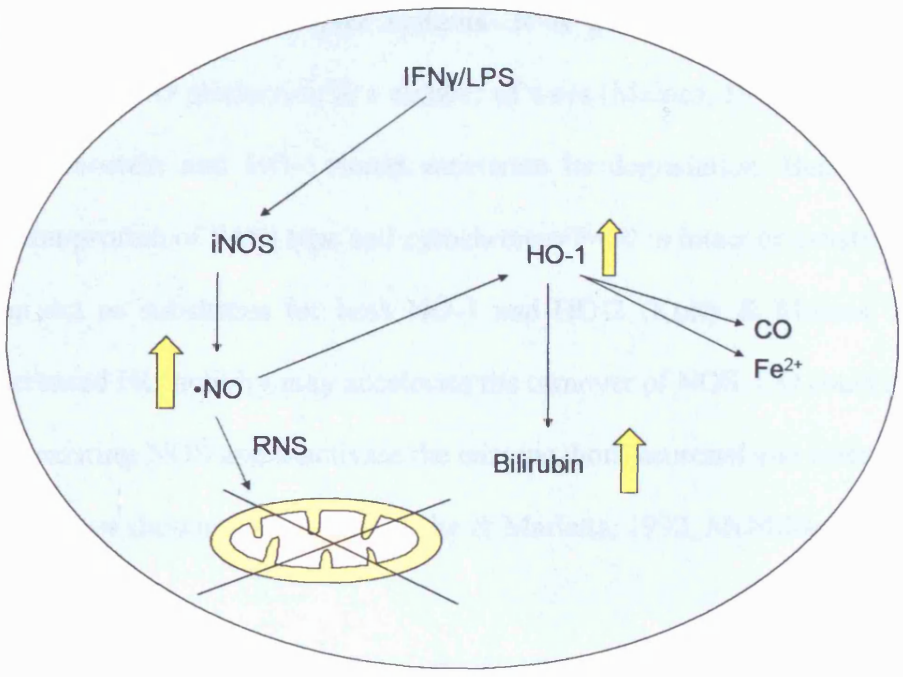
The second part of this thesis focused on manipulating the HO status of astrocytes activated by IFNγ/LPS. The relevance of the induction of iNOS and

NO to neurodegeneration has been well documented (for review see Stewart & Heales, 2003), and this thesis has demonstrated that activating iNOS using IFNγ/LPS also activates the HO-1 pathway giving both enhanced expression of HO-1 protein and an increase in total HO activity (**Figure 5.3**.). It was initially hypothesised that it could be feasible that CO generated by HO activity may play a part in the reversible and irreversible inhibition of complex IV shown by Bolanos *et al.*, (1994) and Brown *et al.*, (1995). HO activity was enhanced and inhibited to determine whether its action was protective or harmful, and certainly co-incubating astrocytes with inducers of iNOS and HO did prove to alleviate the irreversible NO-mediated inhibition of complex IV retaining enzyme activity at control levels. If this was an effect of increased HO activity it may be expected that inhibiting HO activity might make the inhibition more extensive. This was not the case in this investigation. IFNγ/LPS activated astrocytes treated with SnPPIX still showed complex IV inhibition, but not to as great an extent as IFNγ/LPS treated cells alone, although the difference between the two treatments was not significantly different. This makes dissecting out the actual protective mechanism difficult, but does indicate that in these circumstances HO-1 induction is not a cause of further irreversible inhibition to complex IV. As well as preventing the inhibition caused by IFNγ/LPS, adding hemin also caused an increase in GSH levels in the cell. (Schematic:**Figure 6.1**.) This is actually contrary to reports that addition of GSH or its precursors causes a decrease in HO-1 expression and activity (Foresti *et al.*, 1997) since in this thesis the data shows an increase of GSH with an increase of HO activity. It is suggested that this is due to the combined effect of two oxidative stresses – the effect of hemin and that of the HO reaction itself , this is made more plausible by the fact that

astrocytes treated with hemin, IFNγ/LPS and SnPPIX do not show the rise in GSH that occurs when hemin and IFNγ/LPS are used alone. In the former case HO activity is inhibited by SnPPIX thereby removing the majority of a possible ROS generating reaction.

An exogenous source of NO appeared to be much more damaging to the mitochondrial ETC (**Figure 5.13**.) even if HO-1 was induced and despite the fact that treating with DETA-NO and hemin caused GSH levels to rise to a similar extent as seen when treating with hemin and IFNγ/LPS (**Figure 5.7.** and **Figure 5.16.**). As discussed in chapter 5 this may be as a result of the very fast reaction between the NO and bilirubin (Kaur *et al.*, 2003) or the fact that haem degradation products are able to modulate the action of NO donors by accelerating and increasing the release of NO as suggested by Foresti *et al.*, (2003), thus depleting this anti-oxidant too quickly for protection to occur. It is also relevant that using an NO donor almost immediately exposes astrocytes to NO at a concentration that may not be actually occuring inside the cell even though electrode studies have shown comparable concentrations under experimental "ideal" conditions (Brown *et al.*, 1995). For maximal NO production when iNOS is stimulated L-arginine must not be limiting and all required co-factors must be available . (Alderton et al., 2001; Aktan, 2004). NO release by DETA- NO may represent an overwhelming nitrosative stress as demonstrated by the fact that after 24 hours there is already evidence of inhibition to complex II/III, which is not seen in IFNγ/LPS treated cells until 36 hours of treatment (Bolanos et al., 1994) and occurs without the activation of iNOS which could be relevant since there are postulated to be interactions

a)



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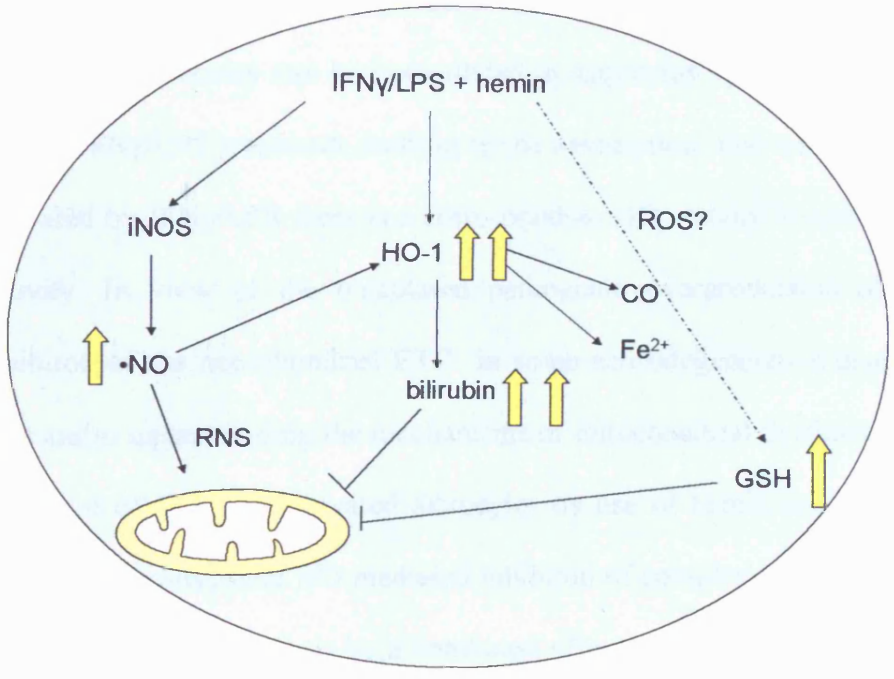

Figure 6.1. Proposed schematic for events occurring when astrocytes are treated with a) IFNγ/LPS alone or b) IFNγ/LPS + hemin.

In a) HO-1 is induced and activity is stimulated but inhibition of complex IV of the ETC occurs. In b) HO-1 activity is more significantly raised , GSH levels are raised and protection is afforded.

between the NOS and HO systems. It is possible that induction of HO-1 modulates NO production in a number of ways (Maines, 1997). NOS is a haemoprotein and HO-1 could accelerate its degradation. Because NOS is a haemoprotein of P450 type and cytochromes P450 in intact or denatured form, can act as substrates for both HO-1 and HO-2 (Kutty & Maines, 1984) then increased HO activity may accelerate the turnover of NOS. CO could also bind to the existing NOS and inactivate the enzyme, both neuronal and macrophage NOS have been shown to bind CO (White & Marletta, 1992; McMillan *et al.*, 1992).

In conclusion the work in this thesis has demonstrated a robust method for measuring HO activity in astrocytes. It has been demonstrated that HO-1 protein and total HO activity can be upregulated in astrocytes by both hemin treatment and by IFNγ/LPS treatment, leading to the assumption that when astrocytes are activated by IFNγ/LPS there is a corresponding HO activity in addition to iNOS activity. In view of the postulated pathogenic overproduction of NO and inhibiton of the mitochondrial ETC in some neurodegenerative diseases this is relevant to understanding the mechanisms of mitochondrial dysfunction. Further induction of HO-1 in activated astrocytes by use of hemin has been shown to prevent the irreversible NO mediated inhibiton of complex IV, and this apparent protection is postulated to be a combined effect of hemin mediated HO action and GSH upregulation. Inhibition of HO in IFNγ/LPS stimulated cells does not return complex IV to control levels therefore it can be postulated that in this instance HO does not have a part to play in the NO mediated inhibiton of complex IV in rat astrocytes. Certainly though, interactions between the systems appear to occur and further study of these interactions will enable a greater

understanding of the mechanisms of neurodegenerative disease and the role that HO plays.

Suggested future work

The work in this thesis has concentrated on induction of various enzyme pathways via chemical means, to complement this it should be possible to approach the problem from a more molecular biological perspective. Overexpression of HO-1 has been demonstrated in rat astroglia by transient transfection of the human HO-1 gene (Schipper, 1999). By using over-expression of HO-1 rather than further induction using hemin it may be possible to remove any "oxidant" effects that hemin may exert. On a similar note since the use of HO inhibitors can have non-specific effects (Grundemar & Ny, 1997; Jozkowicz *et al.*,2003) the use of small interfering RNA could be employed to knock-down the HO-1 gene and examine astyrocytic responses to iNOS induction.

Transgenic HO-$1^{-/-}$ mice have also been produced (Poss & Tonegawa, 1997; Kapturczak *et al.*, 2004), and these could also be a useful tool to examine further the effects of iNOS activation on astrocytes or indeed the whole brain.

Using knock outs could also be used to examine the contribution of the products of haem breakdown to either protection or damage of the ETC. Recently much research has focused on physiological roles for CO (for review see Ryter *et al.*, 2004) including signalling and anti-infammatory effects and further to this a group have synthesised CO releasing molecules (Motterlini *et al.*, 2003) which will greatly aid investigations in this area.

Work in this lab utilises a astrocyte-neurone co-culture system to study astrocyte neurone interactions and inducing HO-1 in astrocytes, then culturing them with neurones may enable us to investigate if astrocytes can be protective via the HO pathway, and facilitate elucidation of the mechanisms by which protection may occur.

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Abstracts and Publications

Invited speaker at the 2nd International Conference on Heme Oxygenase (HO/CO), Catania Sicily June 2002. Heslegrave A., Stewart V., Sharpe M., Calabrese V., and Clark J. (2002) Haem oxygenase expression and activity in astrocytes. *J. Exp. Biol.* (in press)

Poster Presentation at Biochemical Society meeting, Edinburgh April 2002. Abstract: Heslegrave A., Stewart V,. Sharpe M., Calabrese V., and Clark J (2002). Haem oxygenase/Nitric oxide synthase interactions: A role in neurodegeneration? *Biochem. Soc. Trans.* 30: A84